Biota Environmental Sciences

Helix Molecular Solutions

Pilbara Olive Python Monitoring Western Ridge, Ophthalmia Dam and Millstream: 2022-2023

Prepared for BHP WAIO

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Pilbara Olive Python Monitoring 2022-23

Contents

Tables

Figures

Plates

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1.0 Executive Summary

BHP Western Australia Iron Ore (BHP WAIO) commissioned Biota Environmental Sciences (Biota) and Helix Molecular Solutions (Helix) to establish a robust and repeatable Pilbara Olive Python (*Liasis olivaceus barroni*) (POP) monitoring program at Western Ridge and using the regional reference sites of Millstream and Ophthalmia Dam. The POP is currently listed as Vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* and *Biodiversity Conservation Act 2016*.

The broad objective of the POP monitoring program is to understand the population demographics (e.g. change in numbers, health or age / sex structure), habitat usage and movement (spatial ecology) in the eastern Pilbara. The current monitoring period presented within this report represents the first two years of the monitoring program and was conducted over six sampling phases between January 2022 and February 2023.

Methods utilised to detect and monitor POP, including targeted searches, mark-re-capture, radio-telemetry, motion cameras, tissue collection and genetic relatedness analyses, and environmental DNA (eDNA) sampling of rock pools.

The program involved collaboration between Biota, Helix, Department of Biodiversity, Conservation and Attractions Senior Research Scientist and POP researcher Dr. David Pearson, Murdoch University Associate Professor Dr. Peter Spencer, Wattle Grove Veterinary Hospital wildlife veterinarian Dr. Samuel (Timothy) Oldfield and BHP WAIO staff.

Population Demographics
Twenty-eight POP individuals were detected during the monitoring program; comprising seven individuals at Western Ridge, 15 at Ophthalmia Dam and six at Millstream. Twenty-four of the 28 were hand-captured alive, while the remaining four were identified by genotyping sloughs and deceased remains (two at Western Ridge and two at Ophthalmia Dam). An additional slough collected at Western Ridge was found to be a genetic match to an already captured individual from that site.

The sex ratio of live captured animals (n=24) was broadly even, comprising 11 males and 13 females with the ratio at each site as follows: Western Ridge, two males and three females; Ophthalmia Dam, seven females and six males; and Millstream, three males and three females. Individuals were categorised into three discernable age classes: juvenile, sub-adult and adult. There was a modest bias towards the capture of adults, with 15 individuals recorded, followed by six juveniles and three sub-adults.

Habitat Use
Of the initial capture events, a relatively small proportion (seven of 24) were recorded directly within water bodies. This finding highlights limitations in the comprehensiveness of eDNA as a tool for monitoring the species, further emphasised by the relocation data from the telemetry studies (see Spatial Ecology below). A seasonal shift in habitat use was observed over the duration of the program, wherein during the hotter, wetter months, animals inhabited water bodies, whereas in the cooler, dry months, they moved to rock crevices and boulder piles likely representing brumation sites. Many of these sites had a predominantly northern aspect and warmer temperatures compared to similar southern-facing habitats. The geographic extent of the habitat shift was observed as most pronounced at Ophthalmia Dam, where rocky habitats are located several kilometers from water, unlike the closer proximity at Western Ridge or Millstream. Even during the wet season, animals were not strictly confined to water bodies. For example, at Western Ridge, with the exception of disturbed/cleared habitats, POP were tracked across all present habitats, including stony plains, mulga woodland, and drainage area/floodplain habitats. At Ophthalmia Dam, POP were also recorded using open spinifex plains, which are not typically considered habitat for the species.

Spatial Ecology
Twenty-four POP were radio-tracked during the program, which represents the first industry-based tracking program for the species.

The radio-tracked POP were typically well hidden; beneath rocks and rockpiles, in small holes, deep within crevices, or within thick vegetation. Many of the tracked POP had moved significant distances and ranged throughout dry ridges, gorges and gullies over the course of the study. These findings align with previous research, which found that POP undertake significant movements in short spaces of time and have home ranges of up to 4.7 km^2 on the Burrup Peninsula.

Radio-telemetry showed that the likelihood of relocating a POP through visual targeted search is very low, even if the animal is in the searched area. During the 34 survey days/nights at Western Ridge and Ophthalmia Dam, none of the POP were re-detected by targeted searches alone. All were re-detected only by radio-tracking.

eDNA Sampling
The eDNA samples analysed detected POP at all sites, with varying degree of efficacy. Observations from monitoring to date indicate that any targeted search or eDNA sampling program is likely to underestimate the true occupancy and distribution of POP at a given site. This calls into question the effectiveness of this technique as a standalone in any such monitoring program.

Preliminary results from the species-specific probe-based quantitative PCR, which was tested in Phases 5 and 6, compared favourably with the 16S metabarcoding approach typical of current POP eDNA studies. In Phase 6 (Millstream), no samples analysed with the 16S metabarcoding method tested positive for POP, while six of the same samples did when assayed with the qPCR probe. This initial result indicates that the species-specific probe offers superior detection capability for the target species.

Genetic Kinship Studies
Genetic kinship studies found none of the populations to be inbred, and found evidence for historical connections between the sites, with stronger connections between Western Ridge and Ophthalmia Dam, than either of those sites to Millstream. It also found direct familial relationships between several of the captured POP, including parent-child and full sibling relationships.

2.0 Introduction

2.1 Project Background

BHP Western Australia Iron Ore (BHP WAIO) commissioned Biota Environmental Sciences (Biota) and Helix Molecular Solutions (Helix) to establish a robust and repeatable Pilbara Olive Python (*Liasis olivaceus barroni*) (POP) monitoring program at Western Ridge and regional reference sites. The program was commissioned for an initial two-year monitoring period.

The broad objective of the monitoring program is to understand the population dynamics (e.g. change in numbers, health or age / sex structure), habitat usage and movement of POP in the eastern Pilbara, with particular focus on Western Ridge. Specific to Western Ridge, the program seeks to improve the understanding of POP population abundance/dynamics and habitat usage within Nankunya (a narrow gorge supporting a system of pools/seeps) and the nearby gorge/gully habitat at Western Ridge. The program is intended to build on the findings of a previous targeted fauna survey (Biologic 2020) and a Matters of National Environmental Significance (MNES) fauna study (Biologic 2021) conducted at Western Ridge.

Upon consultation with Department of Biodiversity, Conservation and Attractions (DBCA) Senior Research Scientist and POP researcher Dr. David Pearson, and BHP WAIO, Millstream-Chichester National Park (Millstream) was selected as a suitable reference site (Figure 2.1). Early in the program, COVID-19 regulations restricted access to Millstream, and Ophthalmia Dam, near Newman, was added as a second reference site (Figure 2.1).

2.2 Scope of Works and Objectives

The scope of this study was to design and implement the first two years of a POP monitoring program at Western Ridge and appropriate reference sites, with the program's specific objectives including:

1. The design and implementation of a monitoring program including targeted searches, mark-recapture, radio-telemetry, remote cameras, genetic kinship analysis, and environmental DNA (eDNA) sampling and analysis.
2. Placing emphasis in the design on investigating the probability of detection of POP across several different monitoring methods and reviewing the implications of this for species detection.

Preparation of a report on the first two years of the program, including project background and objectives, information on POP, detailed methodology, results and analysis, discussion, figures, references and appendices.

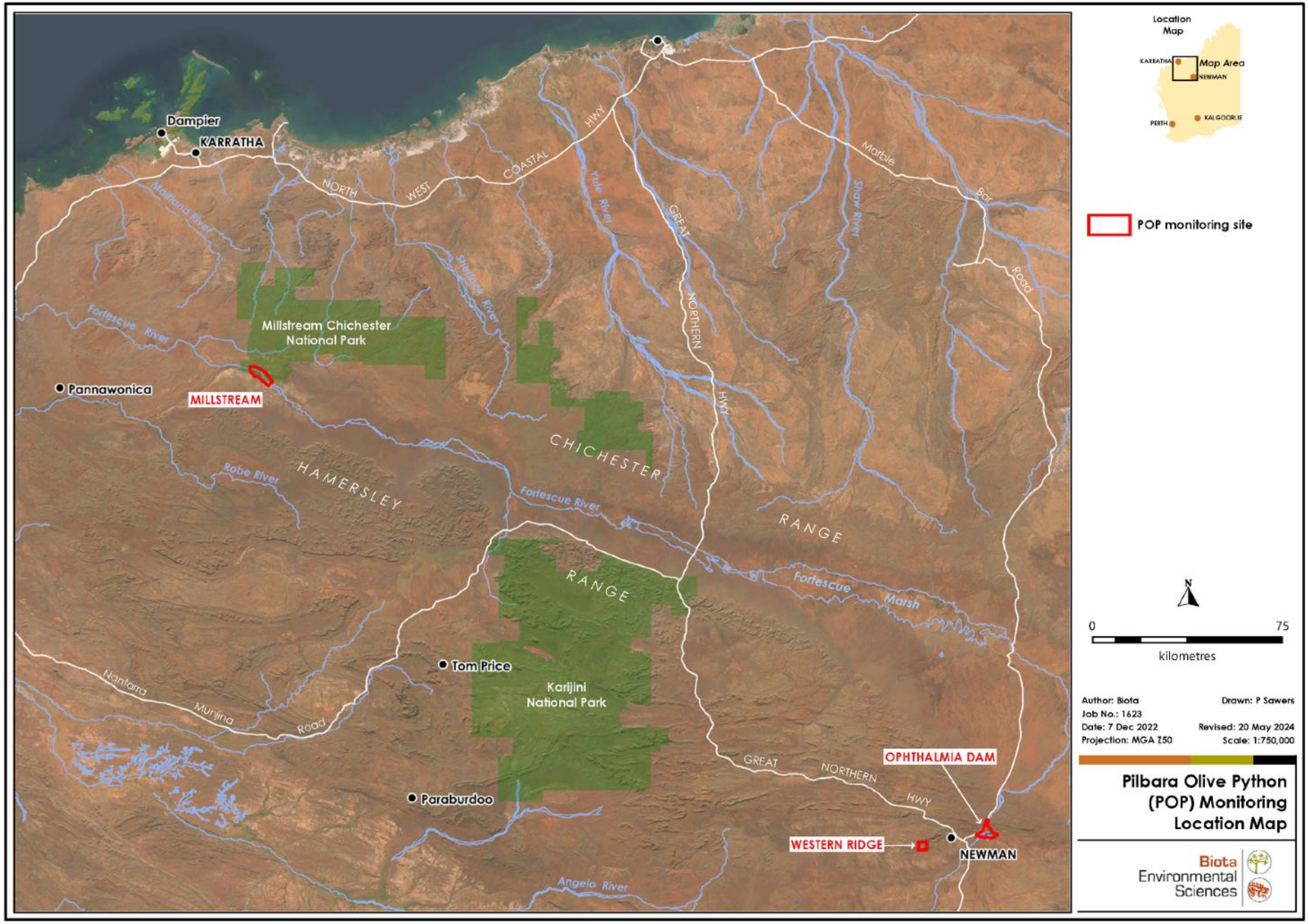

Figure 2.1: Regional location of the monitoring sites.

3.0 Background on the Species

3.1 Current Taxonomy

Family: Pythonidae

Scientific Name: *Liasis olivaceus barroni*

Synonym (non-current): *Morelia olivacea barroni*

Common Names: Pilbara Olive Python; Olive Python (Pilbara subspecies)

Sister taxon: *Liasis olivaceus olivaceus* (Olive Python; northern subspecies)

The Pilbara Olive Python was described by Smith (1981) based on just eight individuals. It was distinguished from the Kimberley populations from differences in the number of mid-body scale rows and ventral scale counts (Smith 1981). Genetic studies have indicated that *L. o. barroni* warrants elevation to full species, but this is awaiting complementary morphological appraisal and publication (Pearson et al. 2013). Phylogenetically, the genus *Liasis* (which contains three extant species) is embedded among the Australo-Papuan python genera, being most closely related to the *Apodora* pythons of Papua New Guinea (Rawlings et al. 2008).

3.2 Description

POP are a dull olive-brown to pale fawn or rich brown python, with a white/cream belly, pale lips finely dotted with pale grey or brown, pitted anterial scales bordering the lips, and smooth scales in 55–80 rows at mid-body (DCCEEW 2023). In bright light, the dorsal scales can take on rainbow hues. POP generally grow to 4 -5 m in length, with an average size of 2.5 m (Bush and Maryan 2011, DCCEEW 2023), though individuals up to 6.5 m have reported (Wilson and Swan 2021). Females may grow slightly longer than males (DCCEEW 2023). Hatchling POP sizes are largely unknown; however, northern olive pythons, *L. o. olivaceus*, hatched in captivity over several decades have ranged from 630 -695 mm total length (mean 660 mm) and 60 -75 g (mean 67 g) (Sonneman 2023).

3.3 Distribution

POP are distributed throughout the Pilbara bioregion; from the Burrup Peninsula on the coast, north to the Ord Range, inland to the Ripon Hills and south to the Barlee Range, with isolated populations on Dolphin Island and at Mount Augustus in the Gascoyne bioregion (Pearson 1993, Bush and Maryan 2011).

The species is allopatric to the northern Olive Python subspecies, *L. o. olivaceus*, with the two taxa separated by the Great Sandy Desert; a 284,993km^2 bioregion whose habitats are largely unsuitable for both subspecies.

3.4 Habitat

POP are most commonly encountered in habitats with ready access to shelter and freshwater, such as gorges, rockpiles, permanent springs and vegetated watercourses (Bush and Maryan 2011, DCCEEW 2023). They have been recorded sheltering within and beneath boulder piles, rocks, gorges, spinifex tussocks, artificial structures and water bodies; the latter being where they are most often sighted during visual surveys, as they wait underwater; ambushing prey at the water's edge (Pearson 2006; Bush and Maryan 2011; DCCEEW 2023).

3.5 Diet

POP are opportunistic ambush predators, with large heat sensing pits in their lips which enable them to better sense warm-bodied prey. Preferred prey items scale with python body size, and include a wide range of birds (e.g. finches, budgerigars, spinifex pigeons, crested pigeons, corellas and large waterfowl), as well as mammals (e.g. house mice, rock rats, quolls, rock-wallabies and euros), frogs and reptiles (DCCEEW 2023). Adult northern Olive Pythons (*L. o. olivaceus*) have been recorded consuming large snakes and crocodiles. A single meal of an adult Rothschild's rock-wallaby (*Petrogale rothschildi*) is considered sufficient to encourage a successful breeding season for an adult female POP (D. Pearson, DBCA, pers. comm.2022).

3.6 Breeding

There is a paucity of information in the literature regarding the Pilbara Olive Python, likely due to the difficulty in the general detection of the species and the locality of incubation sites, in addition to the species not listed for captivity. The Olive Python, like all Australian representatives of the family Pythonidae are oviparous (egg-laying) (Shine1991). In the Kimberley Olive Python, *Liasis olivaceus olivaceus* or 'KOP', males engage in combat during the breeding season being June, July and August (Sonnerman 2007, 2023). Almost all accepted breeding information comes from captive animals, whereby mating has been observed in May through to mid-July (Sonnerman 2007) and is thought to be required to trigger ovulation. For KOP the average gestation time (period between ovulation and egg-laying) is approximately 85 days, with egg-laying occurring around three months after a successful mating event (Sonnermann 2007, 2023). Most clutches are laid in September, but this has been observed from August through to November. Shine (1991) reports an average of 16 offspring per clutch. Hatchlings averaged 660 mm in total length at hatching (Sonnerman 2007, 2023). In captivity, sexual maturity is attained at 3-4 years, although majority of successful captive breeding events have occurred in KOP specimens 8-12 years of age (Sonnerman 2007, 2023).

Pearson (2006) has documented suspected POP breeding during May/June on the Burrup Pearson and the conclusion of mating season in September at Millstream (Pearson, unpublished). From additional unpublished data, it is thought that for POP eggs are probably laid in November under a large rock slab and incubated for two months, with hatchlings appearing in January (Pearson 2006).

3.7 Movements

POP have previously been radio-tracked in several programs coordinated by DBCA Senior Research Scientist, Dr. David Pearson. This includes the Burrup Peninsula (five individuals; Pearson et al. 2004) and Millstream (a currently ongoing program). In the Burrup Peninsula, home ranges ranged from 87.76 - 449.26 ha, with males having larger home ranges than females (Pearson et al. 2004). This is significantly larger than the average home ranges observed for other large pythons across Australia, including *Morelia spilota imbricata* (17 ha; Pearson and Shine 2002), *M. s. spilota* (17 ha; Slip and Shine 1988), *M. s. mcdowelli* (22 ha; Shine and Fitzgerald 1996); and *Simalia amethistina* (60 ha; Natusch et al. 2022 Natusch and Shine 2022). It is thought that POP may undertake significant movements away from their usual home range whilst mate searching. In some areas, they may also exhibit seasonal shifts in habitat usage; being near water and rock outcrops in warmer months, while occupying caves and rock crevices in the cooler months, particularly those of warmer northwest aspect (D. Pearson, DBCA, pers. comm. 2022).

3.8 Conservation Status

POP is listed under the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EBPC Act) as threatened fauna ("Vulnerable") and as a threatened species on Schedule 2 of the Western Australian *Biodiversity Conservation Act 2016* (BC Act) under "Division 3 - Vulnerable".

These conservation rankings reflect a historical decision to discourage unlawful collection of POP specimens for the pet trade and amateur collectors and were not based on assessment against the IUCN criteria.

The latest assessment of the Olive Python by the IUCN (Doughty et al. (2017); including Roy Teale) assigned a ranking of Least Concern to the Olive Python. A separate assessment of the Pilbara subspecies was not undertaken but Doughty et al. (2017) noted that the Pilbara subspecies was afforded additional protection by both State and Commonwealth legislation. Recent genetic studies concluded the Pilbara population warrants elevation to full species (Section 3.1). Once published, the new species will be evaluated against the IUCN criteria in future, however, in the interim it is likely that the State and Commonwealth will maintain current conservation rankings despite a lack of information required to assess against the IUCN criteria (and which would otherwise possibly see the species listed as Data Deficient). The maintenance of the current conservation rankings means that the species will continue to require monitoring associated with future resource and infrastructure development programs.

An Approved Conservation Advice for the POP was published by the Commonwealth Government in 2008 (DEWHA 2008), and currently remains in effect under the EPBC Act. There is no adopted or made Recovery Plan for POP (DCCEEW 2023).

3.9 Threatening Processes

The Approved Conservation Advice for POP (DEWHA 2008) separates threatening processes into two categories; identified and potential.

The listed identified threats listed by DEWHA (2008) are:

- "*predation by feral cats (Felis catus) and foxes (Vulpes vulpes), particularly of juveniles;*
- *predation of food sources (quolls and rock-wallabies) by foxes; and*
- *destruction of habitat due to gas and mining development (especially on the Burrup Peninsula).*"

The listed potential threats listed by DEWHA (2008) are:

- "*the loss of suitable prey species, particularly in coastal locations where foxes are more prevalent;*
- *deliberate road kills, associated with increased traffic from tourism and industry; and*
- *death resulting from mistaken identification as a poisonous brown snake.*"

In addition, the Commonwealth Species Profile and Threats Database (DCCEEW 2023) lists major fire events and "*further development of mining infrastructure*" as threats to the species' habitat, while also noting that "*additional water bodies such as dams and sewage ponds, associated with mining or development, appear to benefit the subspecies*".

Other potential threatening processes not specifically listed on either the Approved Conservation Advice or the Species Profile and Threats Database, but which may be of significance across the POP's entire range, include:

- habitat degradation by cattle (particularly degradation of riparian vegetation);
- replacement of natural grasses by buffel grass (*Cenchrus ciliaris*);
- an associated increase in the frequency and intensity of fires fuelled by buffel grass, particularly around rock piles or riparian vegetation;
- collection of individuals for the illegal wildlife trade (Pearson et al. 2013); and
- changes in hydrology that results in changes to the water table levels, increased run-off, sedimentation or pollution.

3.10 Research Priorities

The Approved Conservation Advice for POP (DEWHA 2008) lists the following research priorities for the species:

- design and implement a monitoring program;
- more precisely assess population size, distribution, ecological requirements and the relative impact of threatening processes; and
- undertake survey work in suitable habitat and potential habitat to locate any additional populations/occurrences.

Although the research priorities for the species have been outlined, progress towards reaching these is impeded by the challenges inherent in studying POP, namely, their cryptic behaviour as ambush predators, large individual home ranges and difficulties traversing their habitat. Appendix 1 presents the constraints of current monitoring techniques."

4.0 Methodology

4.1 Monitoring Program Design

4.1.1 Monitoring Methods

The monitoring program was designed to employ and contrast the various monitoring methods currently available for POP monitoring. The monitoring methods were deployed within a comparative framework at each of the monitoring sites and included:

1. Targeted Searches
Sites were visited and searched for POP and evidence of POP, both during daylight hours and at night.

2. Mark-Recapture
POP were uniquely marked for future reidentification by both Passive Integrated Transponder (PIT) tag (microchip) and scale clipping.

3. Radio-Telemetry and Radio-Tracking
POP (of suitable size) were fitted with internally-implanted very high frequency (VHF) transmitters, allowing each animal to be more easily relocated. This also allowed us to critique the effectiveness of Targeted Searches and Mark-Recapture at re-detecting individuals at each site.

4. Motion Cameras
Motion cameras were deployed at Western Ridge at locations possibly suitable for detecting POP or large macropod prey items.

5. Tissue Collection and Genetic Kinship Analyses
Samples, including tissue from each captured POP, as well as scats, deceased remains and sloughs, were collected for DNA extraction and subsequent genetic analysis. The analyses allowed for levels of genetic relatedness within and between populations from each site to be investigated, including quantification of inbreeding potential, and relationships between individual snakes.

6. eDNA Sampling
Water samples were collected from surface water sources at each site to detect the presence POP eDNA. A species-specific probe-based quantitative PCR (qPCR) assay has been developed for POP (in conjunction with data from a separate BHP POP monitoring program at Yarrie). The results of this new qPCR probe were contrasted to those obtained via eDNA 16S metabarcoding (completed by eDNA Frontiers).

4.1.2 Reference Site Selection

In formal monitoring design, the primary purpose of a reference site, or suite of sites, is to provide a baseline against which changes at impact sites can be compared to distinguish project impacts from natural stochastic variation. Following consultation with BHP WAIO and DBCA Senior Research Scientist Dr. David Pearson, Millstream was selected as the initial reference site for the program. Millstream represented a single large site, with optimal POP habitat concentrated around the Fortescue River and associated springs and tributaries. It also contained suitable accommodation options, at the site's DBCA Ranger station, and has a known large, healthy population (D. Pearson, unpublished data). Incorporating Millstream into the monitoring program would allow for mutually beneficial data collection and sharing between DBCA and BHP WAIO.

Following Phase 1, it became apparent that COVID-19 regulations would restrict regular access to Millstream. As such, Ophthalmia Dam was suggested by BHP WAIO as a potential second reference site. Despite being an artificial wetland, it has permanent water, areas of thick riparian vegetation and ridgelines nearby, as well as historical POP records, and is within 50 km of Western Ridge. With this combination, it was seen as a worthwhile addition to the program.

4.2 Monitoring Sites

4.2.1 Western Ridge

Western Ridge is located approximately 12 km west-southwest of Newman in the Pilbara region of Western Australia (Figure 4.1). Habitats at the site are described in detail in Biologic (2020); and primarily comprise hillcrest/hillslope (69.5%) followed by gorge/gullies (10.6%), stony plains (9.2%; associated with the Mount Whaleback pit), breakaways/cliffs (1.2%), and mulga woodland (0.2%). Of these habitats, gorges/gullies, minor drainage lines, and breakaway/cliffs (totaling 15.8% of the surveyed area) were listed as primary habitat for POP, while hillcrest/hillslope was listed as secondary habitat (Biologic 2020). Therefore, 85.2% of Western Ridge site had previously been identified as POP habitat (e.g. Plate 4.1 and Plate 4.2), consistent with the 13 previous records that have come from within or immediately adjacent to the site (Figure 4.1).

Plate 4.1. **Typical gorge/gully habitat at Western Ridge, dominated by *Triodia, Cenchrus ciliaris,* and eucalypts, with scattered boulders.**

Plate 4.2. **Permanent pool at Nankunya; the only permanent water source at Western Ridge.**

4.2.2 Ophthalmia Dam

Ophthalmia Dam is an artificial waterbody constructed in 1981, upstream of Ethel Gorge approximately 12 km east of Newman (Figure 4.2). The dam supplies water for an artificial recharge system into a nearby aquifer with a borefield for town and mining water supply. The dam is supplied with water by the Fortescue River, which feeds through Ophthalmia Dam into Coondiner Creek and the Ashburton River.

The site encompasses a number of fauna habitats of potential value to POP, including the dam reservoir itself (Plate 4.3), surrounding plains and incised hills and ridges (Plate 4.4), and the drainage systems that feed into the dam. Data supplied by BHP WAIO show two past records of the species from within and immediately adjacent to the monitoring site (Figure 4.3). The area around Ophthalmia Dam has recently been surveyed for fauna by Biologic (in prep.) and detailed fauna habitats are expected to be mapped in that report.

Plate 4.3: **Ophthalmia Dam, showing emergent vegetation, fringing open eucalypt woodland and a dense *Typha* reedbed offshore.**

Plate 4.4: **Incised hills and ridges and plains, both dominated by *Triodia*, surrounding the reservoir.**

4.2.3 Millstream

The Millstream monitoring site is within Millstream-Chichester National Park, approximately 335 km northwest of Newman and 85 km southeast of Karratha. The park encompasses an area of approximately 200,000 ha. However, this site was constrained to the section that intersects the Fortescue River (Figure 4.3). This same area has been the subject of a long-term POP tracking project coordinated by DBCA's Dr. David Pearson (D. Pearson, unpublished data).

The site encompasses permanent spring-fed pools, creeklines, thick fringing reed and riparian vegetation, as well as some gorges and rocky walls, all of which provide suitable habitat for POP (see Plate 4.5 and Plate 4.6).

Plate 4.5. **Fortescue River at Millstream, bordered by low hills and ridgelines.**

Plate 4.6. **Minor drainage lines, heavily vegetated with thick fringing reeds.**

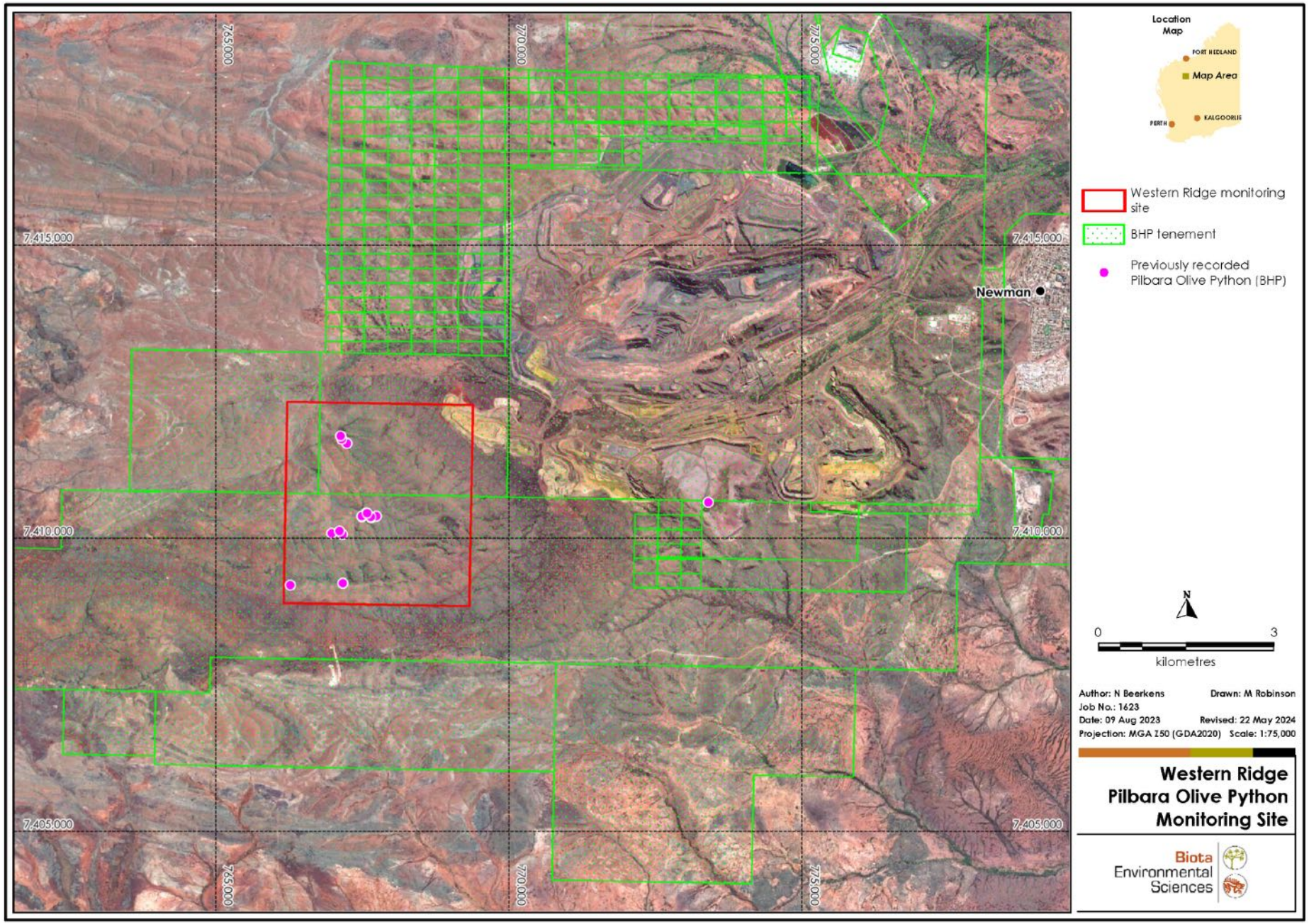

Figure 4.1: Western Ridge monitoring site with previous Pilbara Olive Python records.

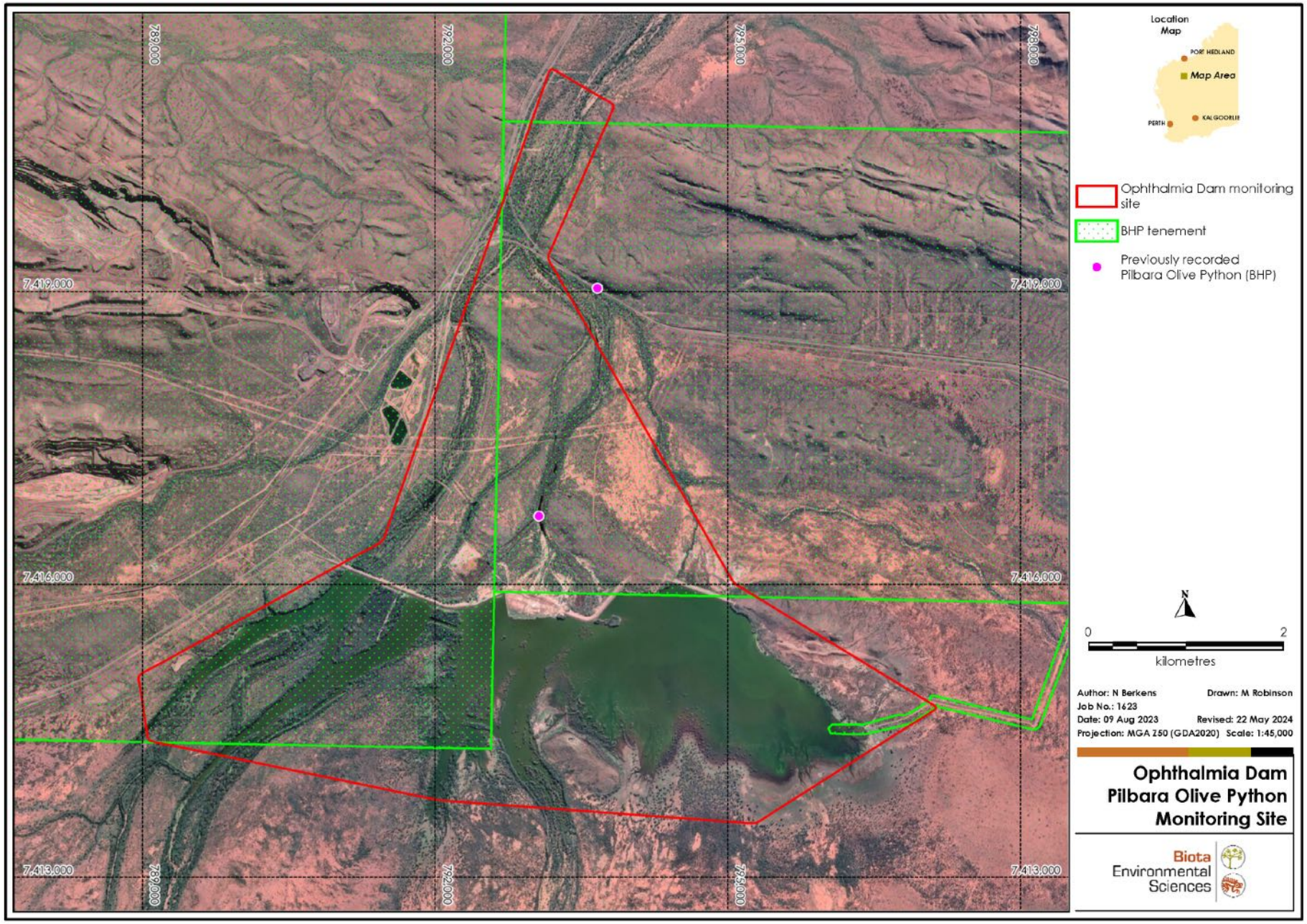

Figure 4.2: Ophthalmia Dam monitoring site with previous POP records.

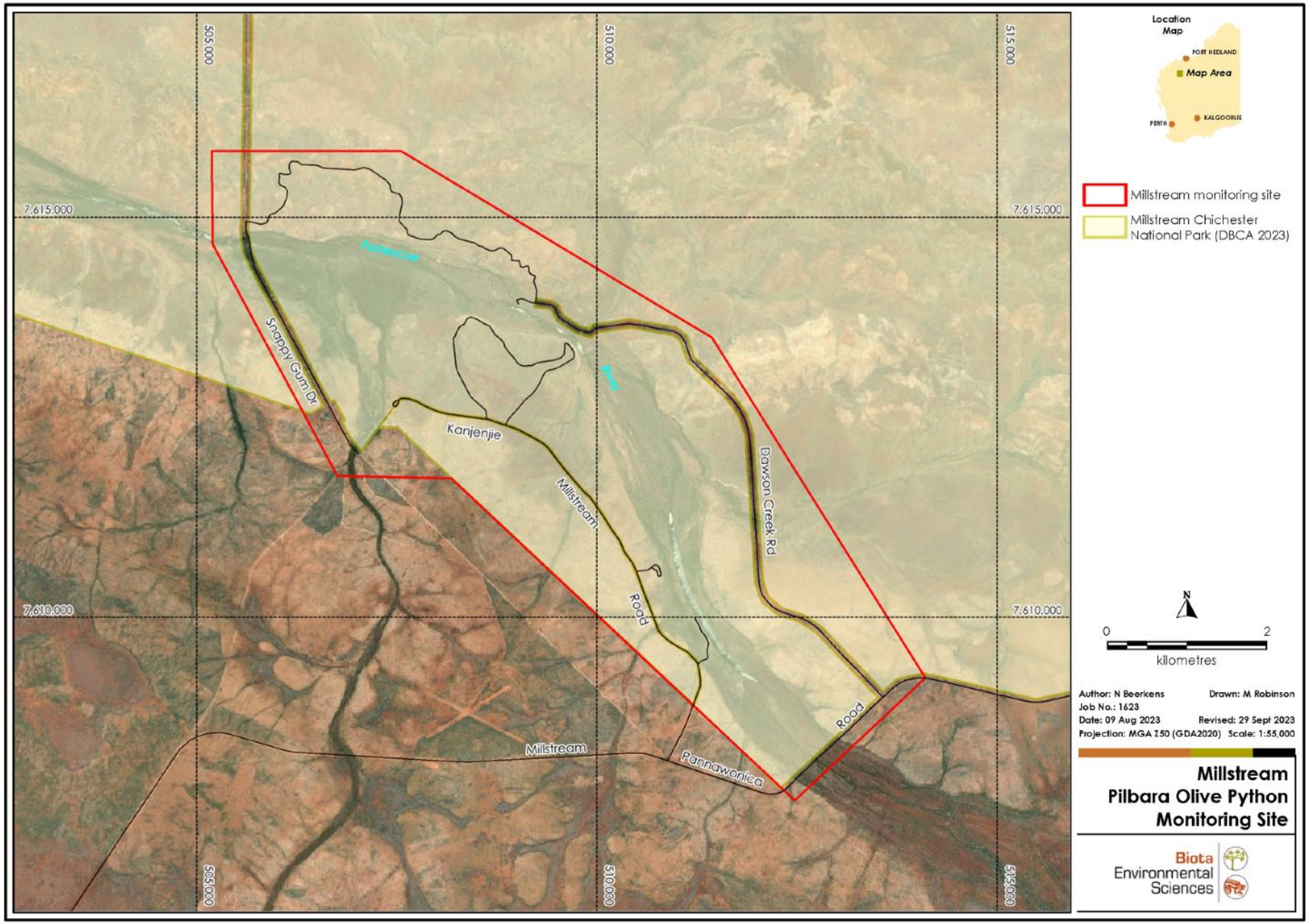

Figure 4.3: Millstream monitoring site.

4.3 Survey Timing and Personnel

This report incorporates data from eleven field deployments (Table 4.1). The six major survey phases were undertaken from January 2022 to May 2023, and are referred to as Phases 1-6. These are the surveys in which pythons were surgically fitted with VHF transmitters, and where genetic samples were taken. Five additional surveys, undertaken by BHP WAIO staff and DBCA's Dr David Pearson, contributed further radio-tracking data to the project (Table 4.1).

Table 4.1. Summary of field surveys undertaken during the monitoring program.

Survey Dates	Survey Activities	Locations	Survey Personnel
Phase 1 11 – 18 January 2022	Targeted searches (nocturnal and diurnal), eDNA sampling, tissue collection, surgical implantation of VHF transmitters, motion camera deployment, radio-tracking.	Western Ridge Millstream	Dr Zoë Hamilton (Helix), Nathan Beerkens, Joshua Keen (both Biota) and Dr Timothy Oldfield (Vet)
Phase 2 22 February – 2 March 2022	Targeted searches (nocturnal and diurnal), eDNA sampling, tissue collection, surgical implantation of VHF transmitters, motion camera collection, radio-tracking.	Western Ridge Ophthalmia Dam	Dr Zoë Hamilton (Helix), Nathan Beerkens, Joshua Keen (both Biota) and Dr Timothy Oldfield (Vet)
Phase 3 7 – 11 December 2022	Targeted searches (nocturnal and diurnal), tissue collection, surgical implantation of VHF transmitters, motion camera deployment, radio-tracking.	Western Ridge Ophthalmia Dam	Dr Zoë Hamilton (Helix), Nathan Beerkens and Dr Timothy Oldfield (Vet)(1day only)
Phase 4 23 – 29 January 2023	Targeted searches (nocturnal and diurnal), eDNA sampling, tissue collection, surgical implantation of VHF transmitters, motion camera collection, radio-tracking.	Western Ridge Ophthalmia Dam	Dr Zoë Hamilton (Helix), Nathan Beerkens, Joshua Keen (both Biota) and Dr Timothy Oldfield (Vet)
Phase 5 10 –14 May 2023	Targeted searches (nocturnal and diurnal), eDNA sampling, tissue collection, surgical implantation of VHF transmitters, radio-tracking.	Western Ridge Ophthalmia Dam	Dr Zoë Hamilton (Helix), Nathan Beerkens, Joshua Keen (both Biota) and Dr Timothy Oldfield (Vet)
Phase 6 22 – 25 May 2023	Targeted searches (nocturnal and diurnal), eDNA sampling, tissue collection, surgical implantation of VHF transmitters, radio-tracking.	Millstream	Dr Zoë Hamilton (Helix), Nathan Beerkens (Biota) and Dr Timothy Oldfield (Vet)
Millstream Additional 01 8 – 22 June 2022	Radio-tracking	Millstream	Dr David Pearson (DBCA)
Millstream Additional 02 12-13 August 2022	Radio-tracking	Millstream	Dr David Pearson (DBCA)
Millstream Additional 03 25 – 26 October 2022	Radio-tracking	Millstream	Matthew Love, Jared Leigh, Tanya Carroll, and Suzi Wild (all BHP WAIO)
Western Ridge Additional 01 8 September 2022	Radio-tracking	Western Ridge	Matthew Love, Jared Leigh (both BHP WAIO)
Western Ridge Additional 02 22 February 2023	Radio-tracking	Western Ridge	Matthew Love, Jared Leigh (both BHP WAIO)

4.4 Permits

Field work was completed under Section 40 Authorisation to Take or Disturb Threatened Species License Number TFA 2223-0177 issued by DBCA (Appendix 2) and Animal Ethics Permit RW3360/21 (Protocol ID 898), issued by the Murdoch University Research Ethics & Integrity Office (Appendix 3).

4.5 Weather and Climate

4.5.1 Western Ridge and Ophthalmia Dam

Climate and weather data for the Western Ridge and Ophthalmia Dam sites were obtained from the Newman Aero weather station (007176), as both are approximately 10 km the Western Ridge and Ophthalmia Dam study areas. Rainfall in the year preceding the first phase at Western Ridge and Ophthalmia Dam was higher than average, totaling 385.6 mm in 2021 compared to the long-term annual average of 270.2 mm (1971 - 2023). Only February and May experienced higher-than-average rainfall, which contributed 25.3% and 58.2% respectively, of the total rainfall. Phase 1 and 2 of the surveys at Western Ridge and Ophthalmia Dam were undertaken in January and February/March 2022, which had higher-than-average mean maximum temperatures (Jan 2022 = 41.2 °C; Feb 2022 = 38.3 °C; Mar 2022 = 35.9°C). During Phase 1 there was 9.0 mm of rainfall on one day which, combined with warm temperatures, was conducive to nocturnal herpetofauna activity.

Phases 3 and 4 were undertaken in December 2022 and January 2023. There was no occurrence of rainfall during the phase 3 survey and a total of 15 mm during phase 4 which accounted for 32.8% of the total month's rainfall (Table 5.2). Rainfall during phase 4 occurred across three separate nights with the greatest rainfall (9.6 mm) occurring on the 24th January (see Table 5.2). No rainfall was recorded during the phase 4 survey (Table 5.2). The region typically receives the majority of its rainfall from December to March, however during the third and fourth phase, lower than expected rainfall was recorded at Newman. In December 2022, the total rainfall recorded for the month was 20.8 mm, marking a 38% decrease from the long-term-average of 33.7mm. Similarly, January 2023 witnessed below-average rainfall, with only 45.8 mm compared to the anticipated 70.2 mm, a 34% decline in rainfall.

Maximum temperatures during phases 3 (40.6°C to 43.1°C) and 4 (34.9°C to 39.1°C) both experienced days above the average maximum temperatures for the months (39.7°C and 38.7°C) (see Table 5.2). Minimum temperatures for these phases were comparatively similar (P3: 23.3°C – 27.4°C, P4: 22.8°C – 27.0°C) and were consistent with the average minimum temperatures (P3: 24.8°C, P4: 25.8°C) for the months.

Phase 5 took place in May 2023 when minimum (7.5°C – 14.8°C) and maximum temperatures (23.1°C – 28.9°C) were comparatively much cooler during the survey period (Table 5.2) than previous phases. May is typically characterized by low rainfall and cooler temperatures, during the survey this was consistent with long term trends, however 0 mm of rainfall was recorded in May 2023, compared to the long-term average of 18.6 mm.

Table 4.2: Daily meteorological observations during surveys at Western Ridge and Ophthalmia Dam.

	Date	Minimum Temperature (°C)	Maximum Temperature (°C)	Rainfall (mm)
Phase 1	15/01/22	30.6	40.8	0
	16/01/22	23.8	40.2	0
	17/01/22	23.3	37.8	0
	18/01/21	22.2	35.1	**9.0**
	19/01/21	25.6	36.0	0
Phase 2	22/02/22	20.1	40.7	0
	23/02/22	22.8	42.1	0
	24/02/22	24.2	42.2	0
	25/02/22	25.9	41.1	0
	26/02/22	22.3	41.4	0
	27/02/22	22.5	42.8	0
	28/02/22	28.8	42.3	0
	01/03/22	278.4	41.9	0
	02/03/22	28.6	38.4	0
	03/03/22	25.4	32.9	0
Phase 3	07/12/22	24.2	43.1	0
	08/12/22	27.4	41.6	0
	09/12/22	23.3	41.4	0
	10/12/22	24.3	40.6	0
	11/12/22	24.4	41.5	0
Phase 4	23/01/23	27.0	37.5	0
	24/01/23	22.8	34.9	**9.6**
	25/01/23	25.5	35.3	0
	26/01/23	26.2	39.1	0
	27/01/23	26.9	38.3	0
	28/01/23	23.8	35.1	3.2
	29/01/23	23.6	35.4	2.2
	30/01/23	25.3	37.0	0
Phase 5	09/05/23	10.2	26.8	0
	10/05/23	10.1	25.4	0
	11/05/23	14.8	28.3	0
	12/05/23	9.2	28.6	0
	13/05/23	9.4	28.9	0
	14/05/23	12.8	23.1	0
	15/05/23	7.5	22.8	0

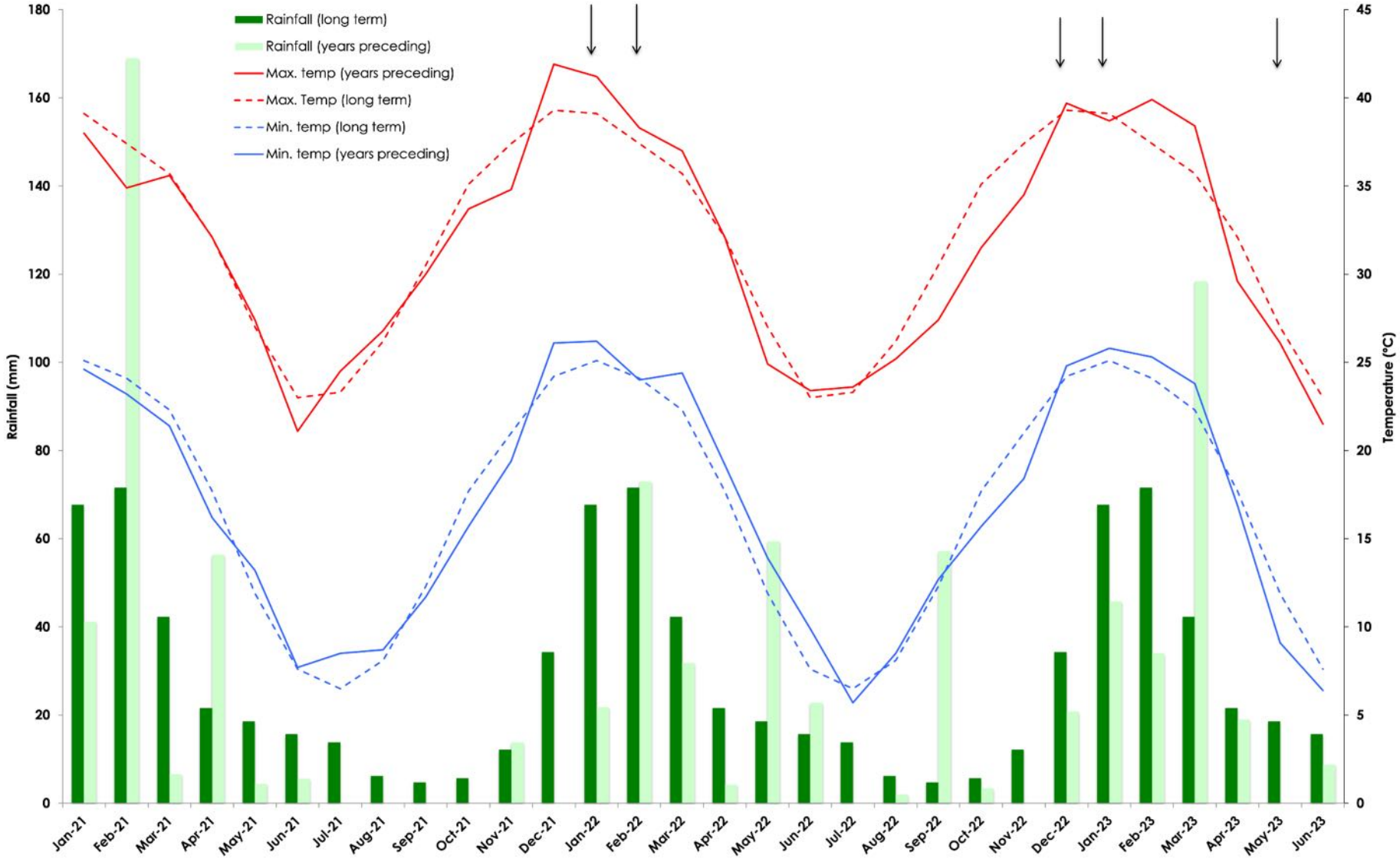

Figure 4.4: **Monthly weather data for the duration of the monitoring program and long-term climate averages at Western Ridge and Ophthalmia Dam.** Arrows indicate survey timing.

4.5.2 Millstream

Meteorological data for Millstream were obtained from the Roebourne Aero weather station (004090), located approximately 50 km north of the monitoring site. As this is a significant separation distance, these data should be interpreted as similar, but not may not be truly representative of conditions at the site. Rainfall at Millstream in the year preceding Phase 1 was higher than average totaling 385.6 mm (long-term average = 286.8 mm). All months presented lower-than-average rainfall, except for February (97.8 mm) and May (224.8 mm) 2021. The monthly mean maximum temperature (42.9 °C) and mean minimum temperature (26.9°C) during Phase 1 were both higher than the monthly long-term average (Figure 4.5). No rainfall was recorded during the surveys (Table 4.3), with significantly lower-than-average rainfall in January 2022 (3.2 mm) and May 2023 (0 mm) (Figure 4.5). The weather during Phase 6 was much cooler, being conducted in May 2023, with lower-than-average mean minimum temperature (14.8 °C) and mean maximum monthly average temperature (30.9 °C) (Figure 4.5). During Phase 1, the area experienced its hottest recorded maximum temperature of 50.5 °C on the 13th of January 2022 (Table 4.3). Although warm weather typically promotes nocturnal herpetofauna activity, these extreme temperatures can suppress movement and result in reptiles entering aestivation in order to conserve water. However, as Millstream has a constant water source in the form of natural springs and the Fortescue River, it is unlikely this would have suppressed POP nocturnal activity.

Dr David Pearson (DBCA) conducted radio-tracking at Millstream in June and August 2023 during which time six pythons implanted with transmitters (in January 2022) were radio-tracked. There was 11.6 mm rain during June 2022, lower than the average amount of rainfall for the month from previous years (30.1 mm). There was less than 1 mm rain recorded during August 2022, which is consistent with the average monthly rainfall (0.7 mm) from previous years. Maximum (27.4°C – June, 29.7°C - August) and minimum (15.6°C and 13.2°C respectively) monthly average temperatures were consistent with the monthly averages from previous years (June min 13.6°C, max 27.9°C; August min 12.7°C max 30.4°C).

Radio telemetry records at Millstream were also obtained by BHP staff (Matt Love and Jared Leigh) in October 2022 (Table 4.1). No rainfall was record in October 2022, less than the 1.3 mm average for the month from previous years. The monthly minimum average for October 2022 (17.7°C was cooler than previous years monthly average (19.6°C). Similarly, the average monthly maximum (34.7°C) was cooler than previous years (37.4°C).

Table 4.3: Daily meteorological observations during surveys at Millstream.

	Date	Minimum Temperature (°C)	Maximum Temperature (°C)	Rainfall (mm)
Phase 1	10/01/22	24.5	37.4	0
	11/01/22	24.5	38.3	0
	12/01/22	28.7	46.1	0
	13/01/22	31.7	50.5	0
	14/01/22	28.3	42.7	0
	15/01/22	31.9	43.4	0
Phase 6	22/05/23	12.6	26.8	0
	23/05/23	8.8	27.7	0
	24/05/23	9.6	-	0
	25/05/23	10.2	31.5	0
	26/05/23	12.9	31.0	0

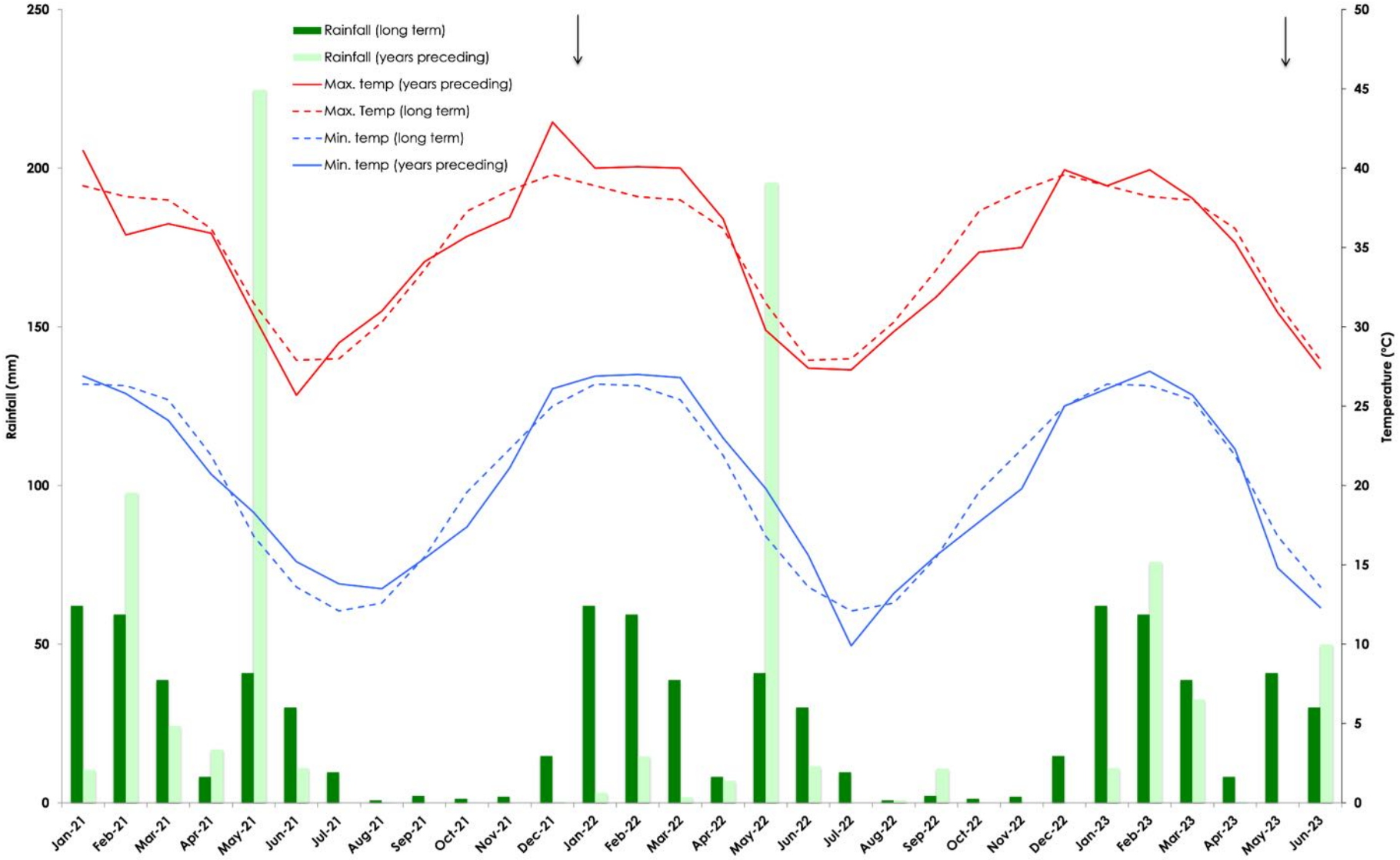

Figure 4.5: Monthly weather data for the duration of the monitoring program and long-term climate averages at Millstream.
Arrows indicate survey timing.

4.6 Targeted Searches and Radio-tracking

Diurnal and nocturnal targeted searches were undertaken to capture POP, collect secondary sign (scats, sloughs and remains), and track previously captured individuals. Nocturnal searches were conducted using high-powered head torches (LedLenser® models). When a POP was located, data regarding its location, habitat, and exhibited behaviour was recorded. The individual was then hand-captured, following the standard operating procedure (SoP) for 'Hand Restraint of Wildlife' (DBCA 2017a) and 'Hand Capture of Wildlife' (DBCA 2017b). The specimen would then be transported to approved processing areas and stored in breathable fabric bags within aerated transport boxes according to the SoP for '*Animal handling and restraint using soft containment'* (DBCA 2017c) and '*Transportation and temporary holding of Wildlife*' (DBCA 2017d).

The total survey effort across all phases and all sites was 295.1 hours, comprised of 123.2 hours at Western Ridge, 104.3 hours at Ophthalmia Dam and 67.6 hours at Millstream (Figure 4.6 to 5.8; Table 4.4). The survey effort expended at each site varied based on accessibility and area of habitat that could be effectively searched. Comprehensive details of the survey effort expended during each phase is presented in Appendix 4.

Table 4.4: Total survey effort during each phase at Western Ridge, Millstream and Ophthalmia Dam.

Survey Phase	Commencement Date	Days	Surveys Conducted	Total Effort (hrs)
Western Ridge				
1	15/01/2022	3	4	19
2	22/02/2022	5	10	41.6
3	07/12/2022	3	3	14.8
4	24/01/2023	3	3	18.1
5	09/05/2023	6	6	29.7
Millstream				
1	11/01/2022	3	7	31.5
6	22/052022	5	5	36.1
Ophthalmia Dam				
2	26/02/2022	4	14	44
3	10/12/2022	1	2	6.8
4	23/01/2023	4	4	24.9
5	09/05/2023	5	6	28.6

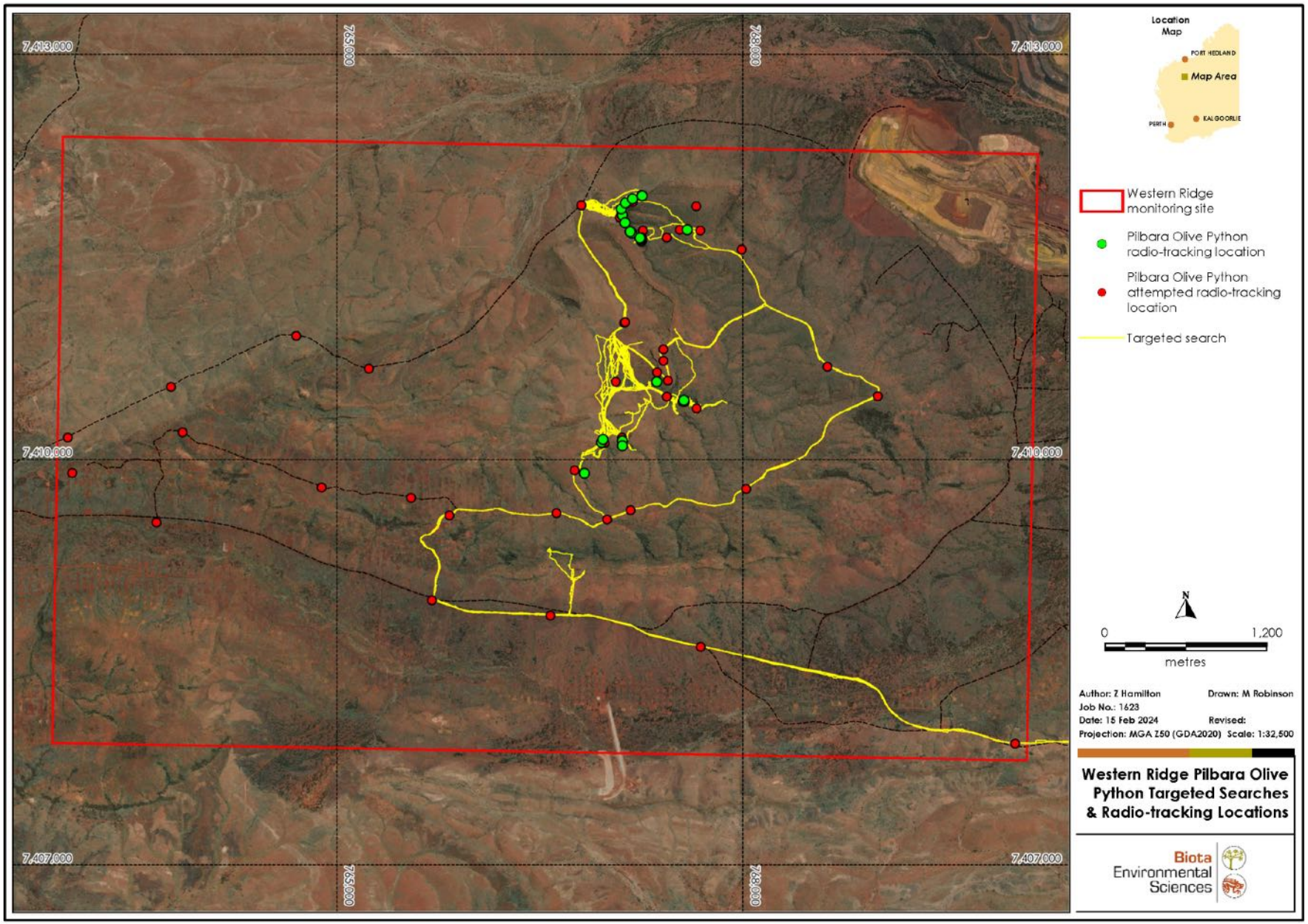

Figure 4.6: Pilbara Olive Python targeted searches and radio-tracking at Western Ridge.

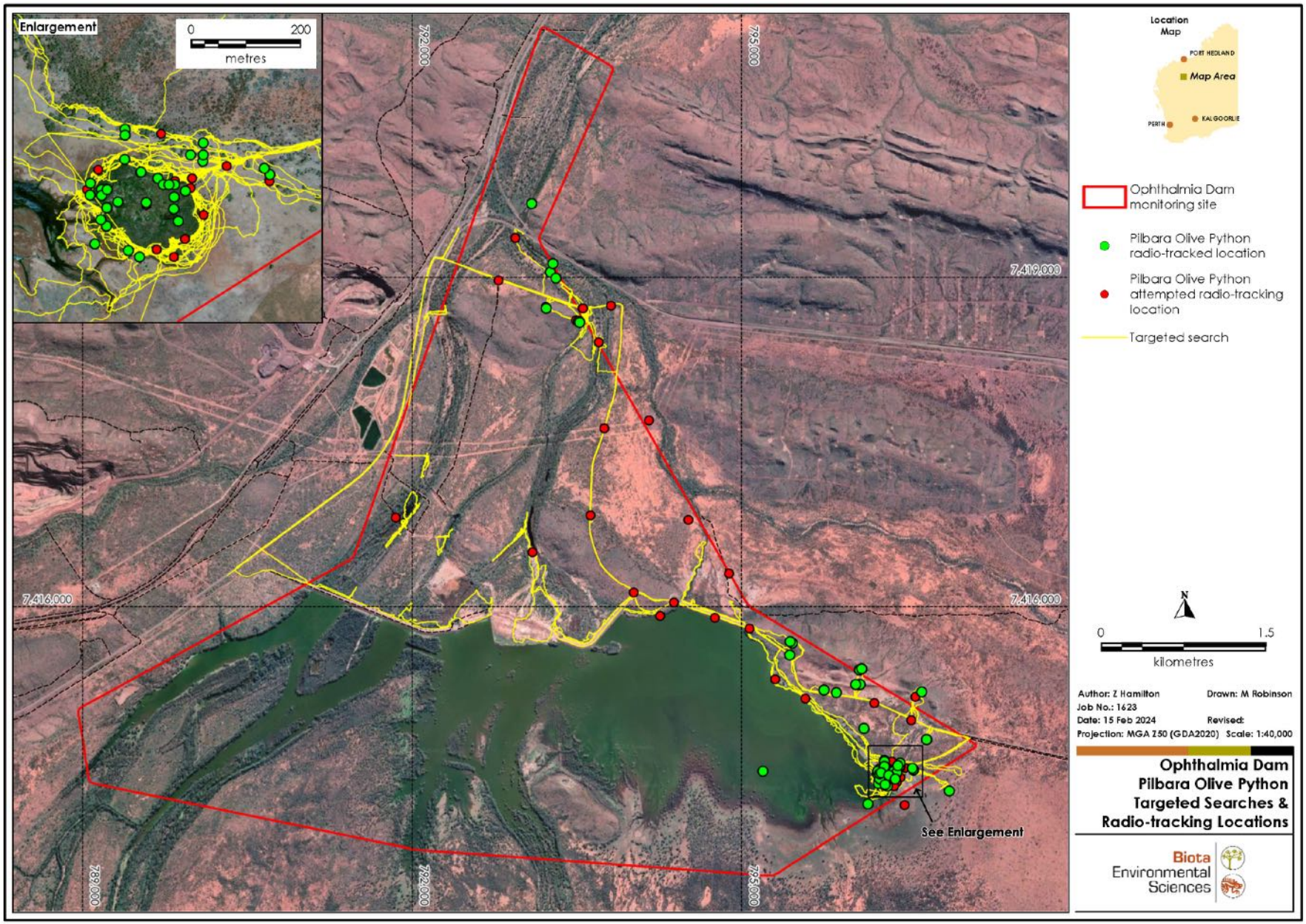

Figure 4.7: Pilbara Olive Python targeted searches and radio-tracking at Ophthalmia Dam.

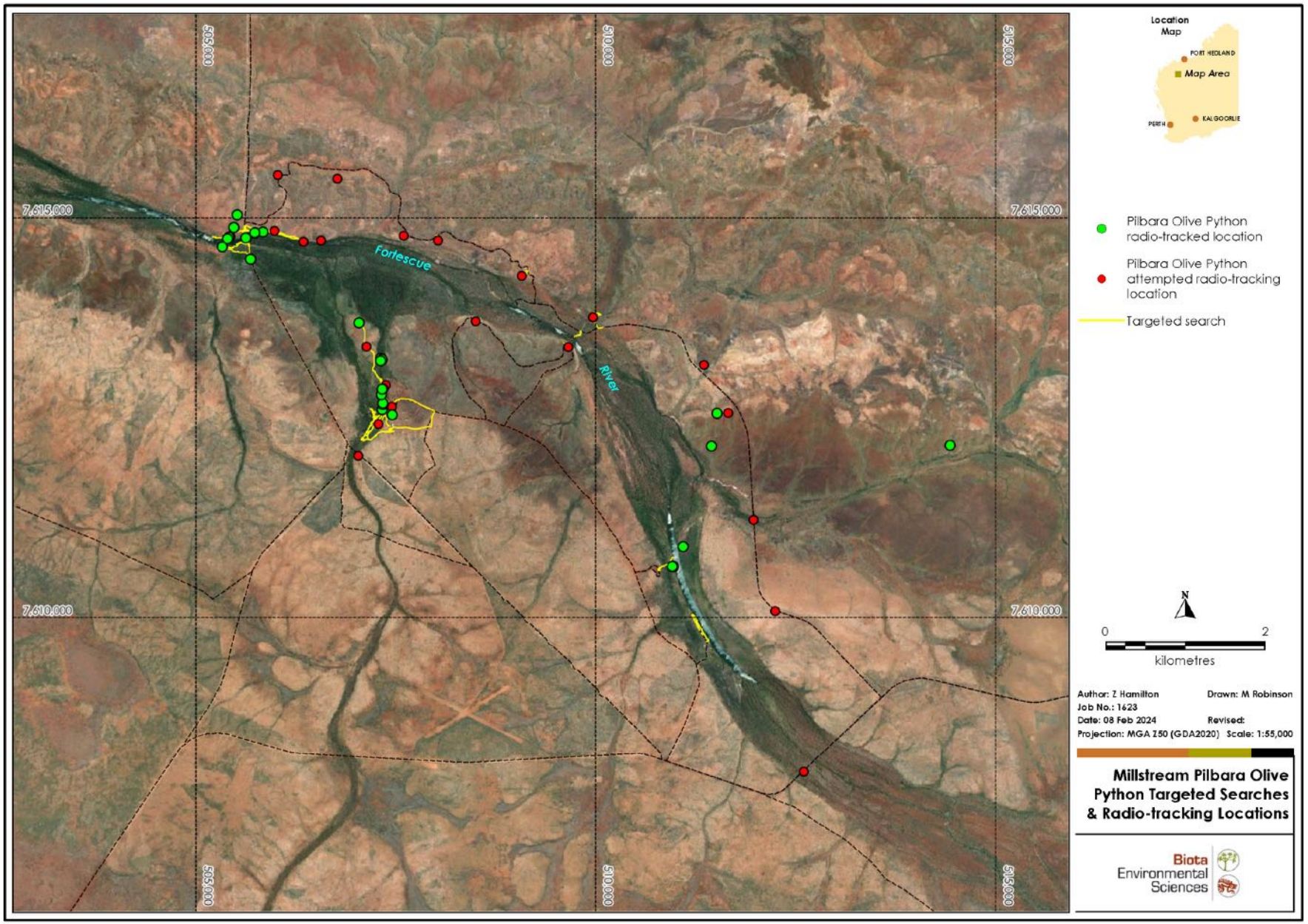

Figure 4.8: Pilbara Olive Python targeted searches and radio-tracking at Millstream.

4.7 Processing of POP

4.7.1 Morphometrics, Meristics and Individual Identification

All captured Pilbara Olive pythons were scale marked in accordance with the SoP for '*Permanent Marking of Reptiles by scale marking'* (DBCA 2017e), and microchipped using Trojan pit-tags in accordance with the SoP '*Permanent marking of vertebrates using microchips*' (DBCA 2017f).

The following data were recorded for each captured POP:

- Weight;
- Snout-vent Length (SVL);
- Tail Length (TL);
- Head dimensions (width x length);
- Ventral and midbody scale counts;
- Sex;
- Body condition;
- Health status;
- Scale clip ID code; and
- Microchip ID code.

Each captured python was uniquely marked via scale-clipping to provide a supplementary form of identification.

Ventral scales above the cloaca on the right-hand side of the python were surgically removed in such a way that would subsequently scar (see Plate 4.7). All scale clipping was performed according to 'Permanent Marking of Reptiles by Scale Marking (DBCA 2017e).

The adjacent two scales were also clipped to avoid misinterpretation of natural injuries and scarring on ventral scales (DBCA 2017a).

The scales and any adjoining tissues removed from the pythons were preserved in 100% ethanol.

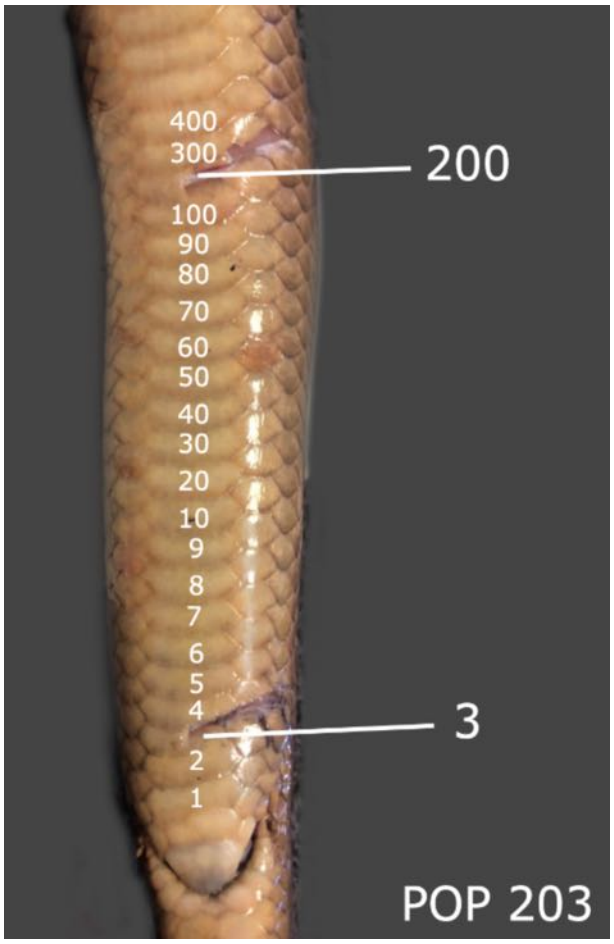

Plate 4.7: Scale clipping demonstrated on POP 203.

4.7.2 Surgical Procedure

To re-detect pythons after they were initially captured, we implanted VHF transmitters into the body cavity of the snake using methods discussed below. Three transmitter models were employed during the monitoring program (Table 4.5; Figure 4.9), all of which were designed by Holohil Systems Ltd (Carp, Ontario, Canada). The size of transmitters varied between models, with battery lifespan increasing with increasing unit size (Table 4.5). Transmitters did not exceed 3.6% of the snake's body mass and in most instances was significantly less.

Table 4.5: Details of the Holohil VHF transmitter units utilised in the study.

Tracker Model	Expected Lifespan (months)	Lifespan Range (months)	Weight (g)	Dimensions (mm)
SI-2T	18	12 – 24	10	40 x 11
SI-2T	24	12 – 30	12	50 x 11
AI-2T	36	24 – 60	28	46 x 17

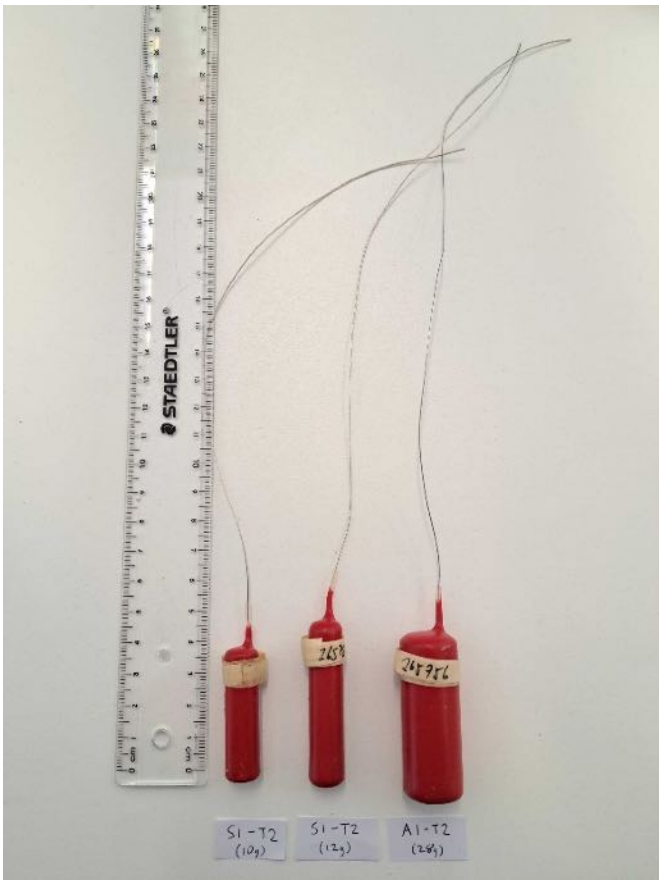

Figure 4.9: VHF transmitters deployed during the monitoring program.

Hand-captured POP had transmitters surgically implanted by a qualified veterinary surgeon using a modification of the methods used by DBCA Senior Research Scientist Dr. David Pearson in his POP tracking projects (Pearson et al., 2004; D. Pearson, DBCA, pers. comm. 2022). Pythons were anaesthetised and operated upon (for the insertion of the radio transmitter) in accordance with the Animal Ethics Permit RW3360/21.

Alfaxan® Multidose (alfaxalone 10 mg/mL) was administered by intramuscular injection at a dose between 4-7 mg/kg body weight (dependent on python size). In all cases, this achieved adequate sedation to facilitate endotracheal intubation after 10–15 minutes. Pythons were then intubated with an appropriately sized, lubricated, uncuffed endotracheal tube. A surgical plane of anaesthesia was attained with manual intermittent positive pressure ventilation (IPPV) at 10 breaths per minute via an Ayres T piece non-rebreathing circuit supplying Isoflurane at 5% in

oxygen at 1 litre per minute. Once induction was complete, anaesthesia was maintained with Isoflurane at 2% - 2.5% in oxygen with manual IPPV 6 breaths per minute. Monitoring of anaesthetic depth was based on visual observation of heart rate and absence of pain response (Plate 4.8). Heart rate and supplied Isoflurane concentration were recorded by a zoologist assisting the veterinary surgeon for the duration of the procedure.

The surgical instruments and the transmitter were soaked in F10™ SC (54 g/L Benzalkonium Chloride, 4 g/L Polyhexamethylene Biguanide Hydrochloride) at a dilution of 1:500 in sterile water for 10 minutes then rinsed in plain sterile water before use.

Location for transmitter implantation was determined by the length of the transmitter and aerial, plus 5 cm, anterior to the cloacal opening. The skin at the site of implantation was prepared with three cycles of alternating iso-propyl alcohol and chlorhexidine 4% w/v scrubs. The surgical site was then isolated by a sterile disposable transparent surgical drape.

A 3-6 cm (depending on the size of transmitter required) incision was made horizontally along the join of the first and second rows of lateral scales above the ventrals (Plate 4.9). The end of the transmitter aerial was introduced into the coelomic space using a specially modified Steinman pin. The Steinman pin was then advanced caudally along the ventral body wall until the aerial was fully inserted. The Steinman pin was then withdrawn. A non-absorbable nylon suture was tied around the body of the transmitter. The transmitter was then inserted into the coelom, the nylon suture was passed around several ribs and tied off to ensure the transmitter was fixed to the internal body wall. The body wall was closed with 2/0 or 3/0 (dependant on size of the python) Polydioxanone sutures in a simple continuous pattern. The skin was closed with the same suture material in a simple horizontal mattress pattern. The closed incision was sealed with surgical grade cyanoacrylate adhesive. Surgical duration averaged 10-15 minutes.

Meloxicam was administered by intramuscular injection at a dose of 0.2 mg/kg and anaesthesia stopped. Manual IPPV with room air was continued at 6 breaths per minute until voluntary respiration recommenced at which point the endotracheal tube was removed. Continuous monitoring was undertaken until a righting reflex was regained. Total anaesthetic duration averaged 60-90 minutes. All animals were strong and active at the time of release. Pythons were released during the evening/night at the site of capture.

Plate 4.8. **Pilbara Olive Python being induced under general anaesthetic.**

Plate 4.9. **Dr. Timothy Oldfield undertaking surgery on a Pilbara Olive Python to implant a VHF transmitter.**

4.8 Motion Cameras

Six Reconyx® Hyperfire 2 motion cameras were deployed at Western Ridge during summer (2022) for a minimum of 46 days (Table 4.6). Cameras were set on breakaways/cliffs and along macropod trackways to target potential macropod prey species, and within Nankunya gorge near evidence of pythons (scats and sloughs; Figure 4.10). Each camera was set to capture three still images per trigger. Total motion camera effort at Western Ridge was 245 camera-nights.

Table 4.6. Location of motion cameras deployed at Western Ridge (Jan-Feb 2022).

Site	Latitude	Longitude	Landform	Deployment Date	Retrieval Date	Days Active
POP01MC	-23.3967	119.6135	Breakaway	16/01/2022	24/02/2022	39
POP02MC	-23.4054	119.6101	Breakaway	17/01/2022	24/02/2022	38
POP03MC	-23.4055	119.6107	Breakaway	17/01/2022	24/02/2022	38
POP04MC	-23.3936	119.6172	Gorge	17/01/2022	23/02/2022	37
POP05MC	-23.3831	119.6146	Gorge	08/12/22	24/01/2023	47
POP06MC	-23.3829	119.6141	Gorge	09/12/22	24/01/2023	46

4.9 Temperature Loggers

Seven temperature loggers (Govee® H5074 Smart Thermo Hygrometers) were deployed during Phases 5 and 6 for two days (Table 4.7). These loggers are capable of recording minute-by-minute variations in temperatures and humidity. Temperature loggers were used during this preliminary trial to record temperatures both inside the location where POP were found to be brumating and immediately outside of the POP retreat, on an exposed area where a POP could bask.

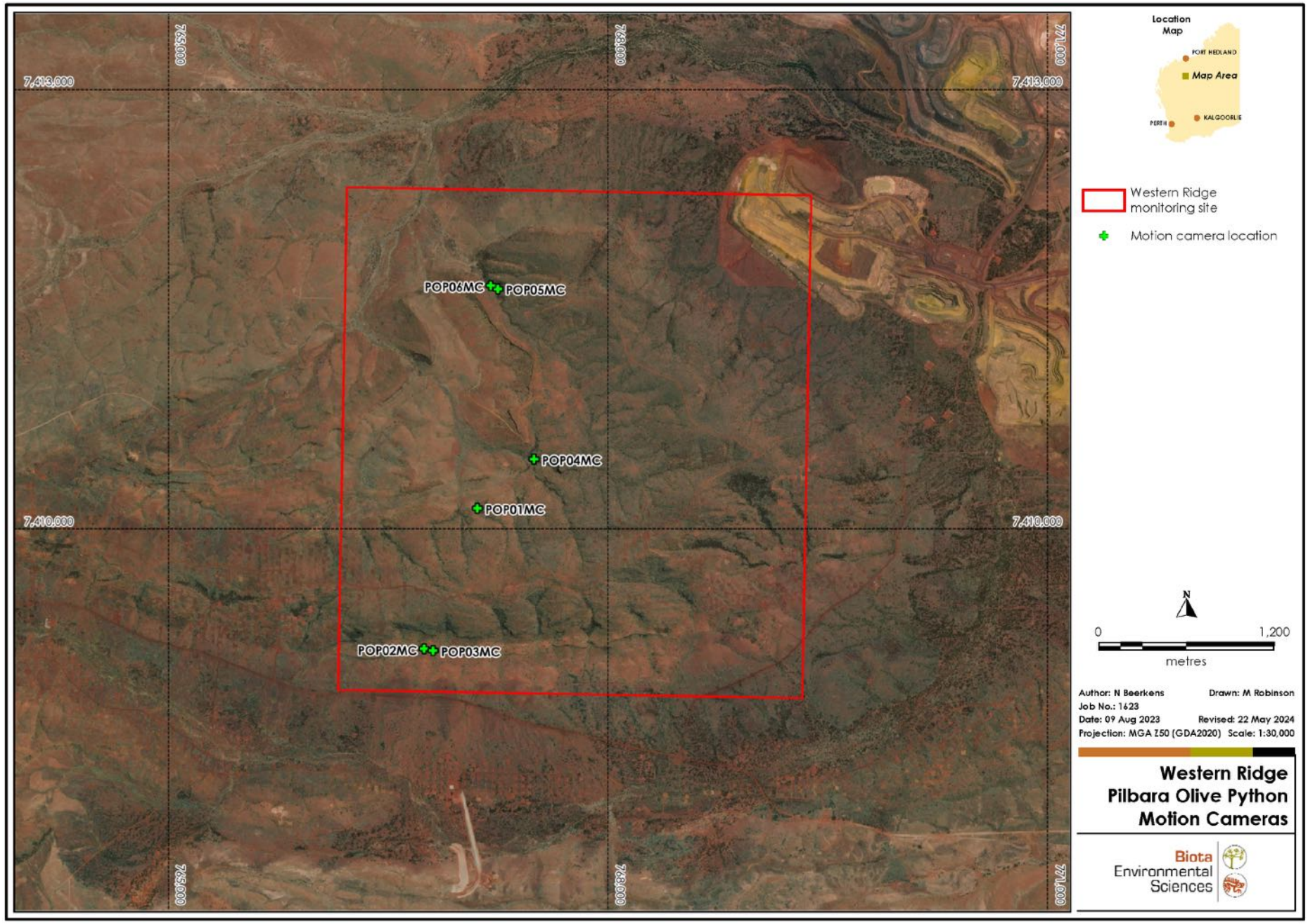

Figure 4.10: Location of motion cameras deployed at Western Ridge.

Table 4.7. Location of temperature loggers deployed during Phases 5 and 6.

Study Area	Location	Latitude	Longitude	Temperature Logger	Deployment Date	Retrieval Date	No. Recording days
Western Ridge	Exposed on a rock, beneath which POP 216 had been found sheltering.	-23.380844	119.613392	1	12/5/2023	14/5/2023	2
	Beneath rock where POP 216 was sheltering. Placed next to POP 216.	-23.380844	119.613392	2	12/5/2023	14/5/2023	2
Ophthalmia Dam	Tucked into northwest side of rockpile, where POP 206 was sheltering.	-23.345695	119.892476	3	12/5/2023	14/5/2023	2
	Tucked into southeast side of rockpile, opposite where POP 206 was sheltering.	-23.345721	119.892522	4	12/5/2023	14/5/2023	2
	Tucked into northwest side of rockpile, next to the sheltering and sloughing POP 207.	-23.349566	119.90173	5	12/5/2023	14/5/2023	2
	Tucked into southeast side of rockpile, opposite where POP 207 was sheltering.	-23.349658	119.901764	6	12/5/2023	14/5/2023	2
Millstream	Tucked into southeast side of rocky hill, at entrance to crevice in which POP 103 was sheltering.	-21.569585	117.052599	7	23/5/2023	25/5/2023	2

4.10 eDNA

4.10.1 Sampling

eDNA sampling took place on five of the six survey phases at Western Ridge (Figure 4.11), Ophthalmia Dam (Figure 4.12) and Millstream (Figure 4.13) (Appendix 5). A water sample was collected from each sampling location using a sterile 1 L container dipped into the water body, while wearing latex gloves. This was repeated twice more to obtain three replicates per site. The samples were kept cool, stored in a lab fridge and then filtered at on-site temporary lab facilities through sterile filter membranes (0.45 µm), using specialist microbiology pumps and funnels. The filters were subsequently frozen, stored in sterile zip-lock bags, individually labelled and then cold-transported to Perth for analysis. Once at Perth, filters were lodged with eDNA Frontiers at Curtin University for metabarcoding analysis, to detect the presence of POP.

During Phases 5 and 6, sample filters were cut in half using a sterile procedure. Both halves were labelled and stored separately, with one half going to eDNA Frontiers for metabarcoding and Helix retaining the other half, to allow a comparison of POP detection success between the eDNA Frontiers 16S metabarcoding method and the Helix POP eDNA probe-based qPCR assay.

A trial temporal study was also performed to attempt to monitor the change in eDNA detection at a single water pool at Western Ridge (Nankunya) over a number of nights. This site was also chosen due to the more permanent nature of the water hole, allowing for repeated sampling, bearing in mind that negative result does not necessarily mean absence of target species, only lack of detection. The pool was sampled over three consecutive nights during Phase 2 and Phase 4, with three replicates collected on each occasion. The site was again sampled in Phase 5 on two occasions, again with three replicates (Appendix 6).

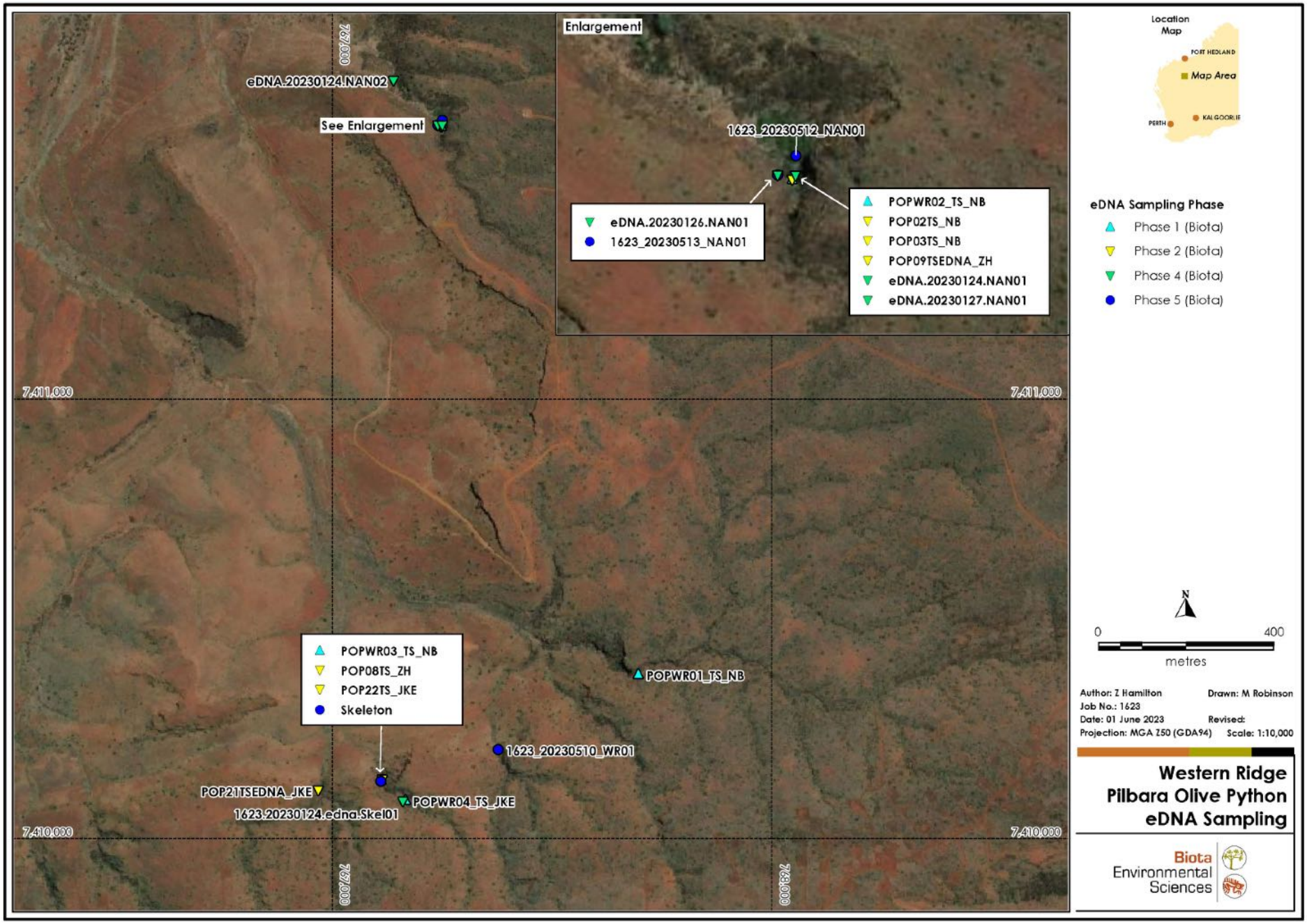

Figure 4.11. eDNA sample locations at Western Ridge, with collection phases coded by colour.

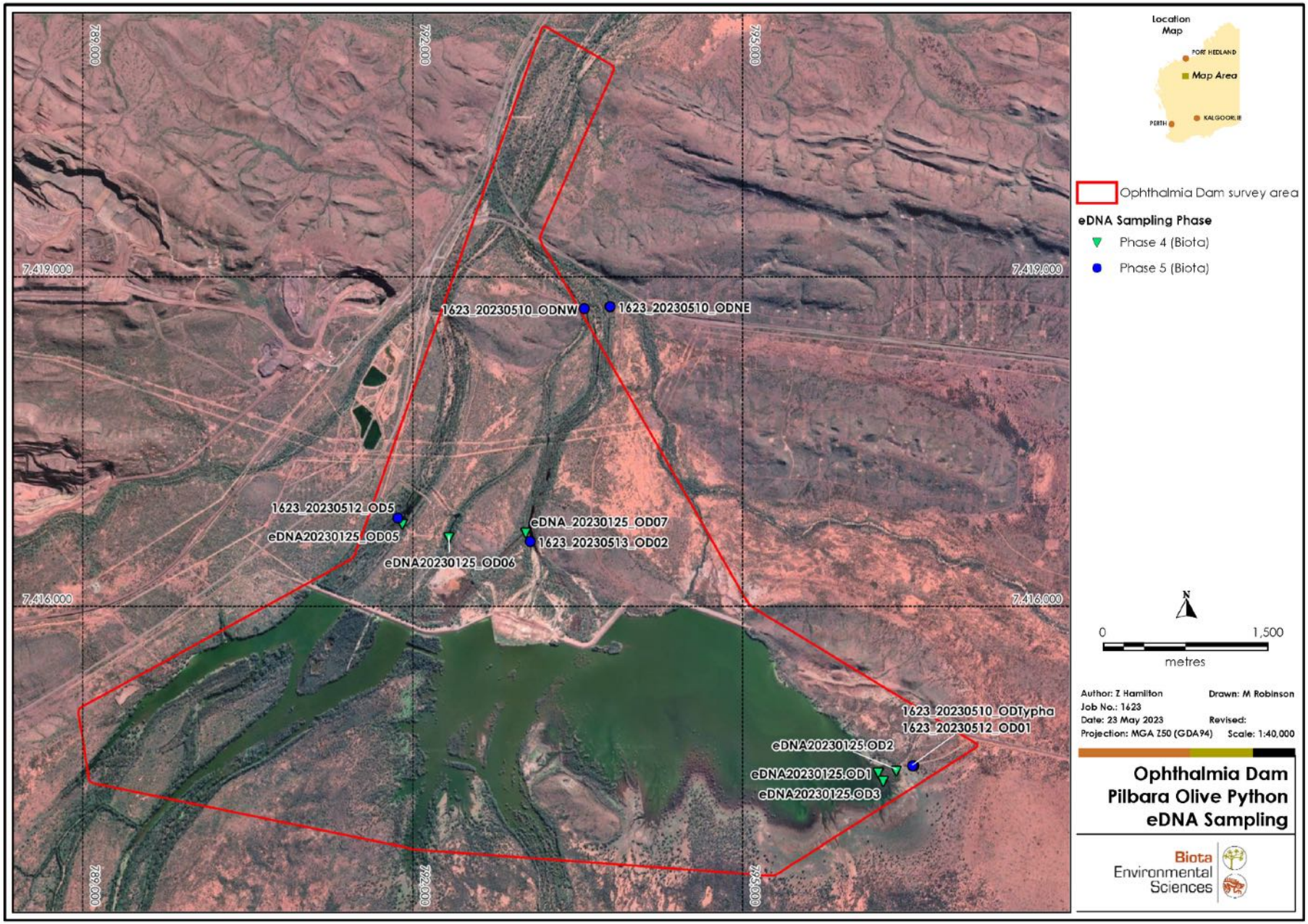

Figure 4.12. eDNA sample locations at Ophthalmia Dam, with collection phases coded by colour.

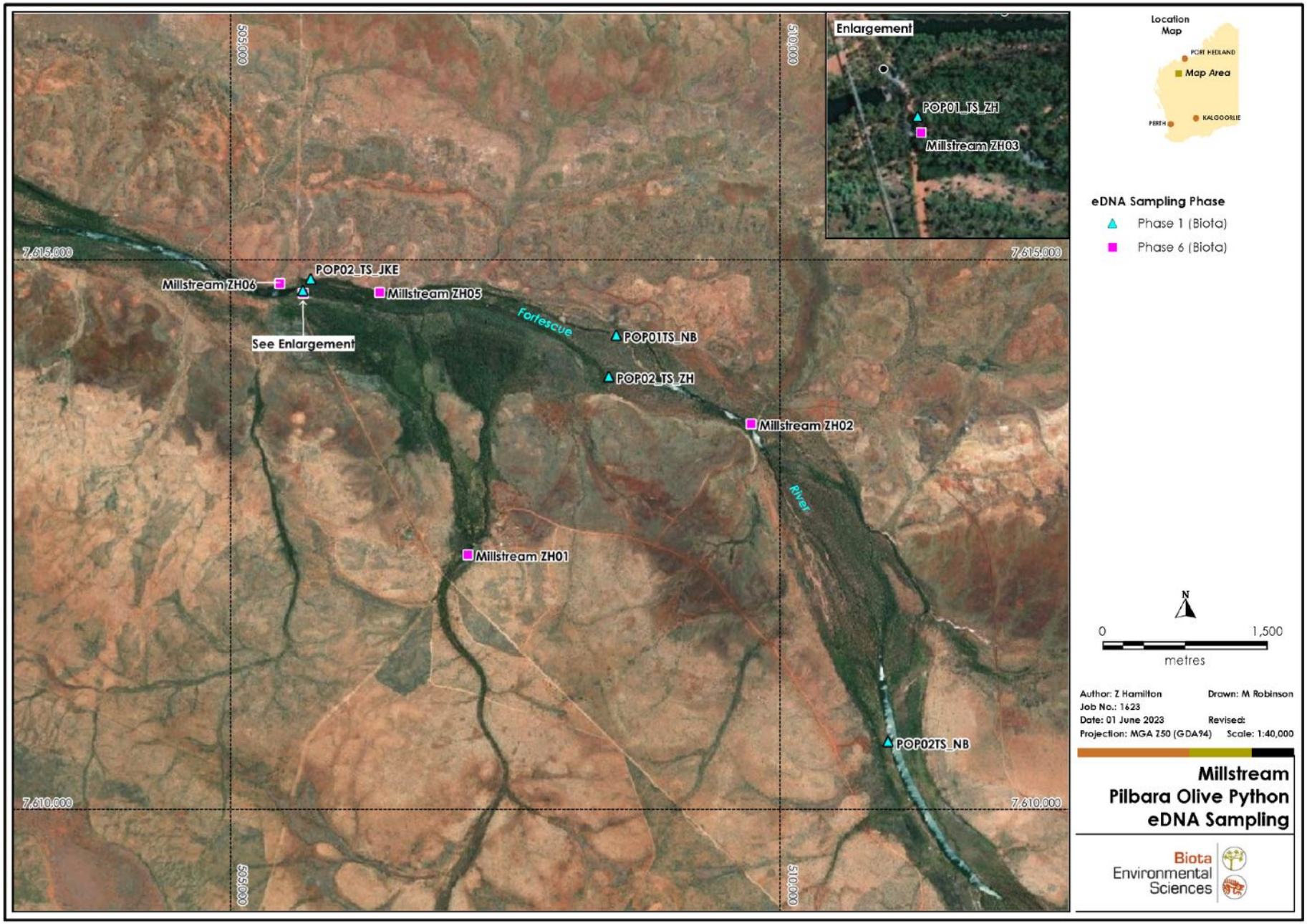

Figure 4.13. eDNA sample locations at Millstream, with collection phases coded by colour.

4.10.2 Analysis

4.10.2.1 Metabarcoding

All individually labelled eDNA filters were submitted directly to eDNA Frontiers at Curtin University for metabarcoding analysis to detect POP, as requested by BHP WAIO.

In total, this comprised:

- 28 filters from 12 sampling locations from Phase 1;
- 46 filters from 16 locations from Phase 2;
- 21 filters from 13 locations in Phase 4;
- 35 filters from 11 sites in Phase 5; and
- 15 filters from 5 sites in Phase 6.

Original eDNA analysis reports from eDNA Frontiers are included in Appendix 6.

4.10.3 eDNA Probe Development and Testing

Helix designed species-specific assays which consisted of two primers and a probe, designed to anneal to specific regions of DNA. The basic requirements for an assay are fragment length, annealing temperature and specificity. Initial design considered two genes; the Cytochrome oxidase I subunit (COI) and Cytochrome oxidase b (CytB) to assess which would be the most specific to POP. Sequence data were obtained for 10 reference species to increase design specificity. Suitability and efficiency of the assays was determined by generating a standard curve using a serial dilution of a known concentration (1 to 0.0016 ng/µl) of *Liasis olivaceus barroni* DNA. The COI assays was deemed efficient with a slope of -3.33 an R^2 value of 0.994 and percentage efficiency of 99.66 and therefore progressed to species specificity trials and detection limits.

The probe was tested on several different python species to ensure cross amplification did not occur which would render the probe uninformative for POP. The probe was tested both on python species that may co-occur with POP (*Antaresia childreni* - R164611 and R173259, *Antaresia perthensis* - R179322 and *Aspidites melanocephalus* - R157604), as well as other python species (*Aspidites ramsayi* - R163452, *Morelia spilota imbricata* - R166918, *Morelia carinata* - R180243, *Simalia amesthestina* R180243, *Liasis olivaceus olivasus* – R164380, R154324 and *Liasis fuscus* - R154325). All tissue specimens were obtained from WA Museum collections, that had corresponding preserved whole specimens to ensure correct species assignment. The assay failed to amplify for these species except those from the genus *Liasis*, which was to be expected due to similarity.

A simplified degradation trial was conducted to determine detection limits and the ability of the assays to amplify from filters. This trial was conducted based off data collected during an associated BHP WAIO POP monitoring program currently being conducted by Biota and Helix at Yarrie. Filters taken at varying time intervals (20 min, 40 min, 60 min, 24 hrs, 48 hrs, 72 hrs and 96 hrs) after the removal of an adult POP from an approximately 25 L artificially constructed pond (large bucket). The assay amplified at all time points in triplicate confirming successful amplification from filters, the sensitivity of the probe and persistence of DNA within a closed environment. Testing was further continued to include two known POP scat specimens which also successfully amplified.

The eDNA probe was then tested on filters from the last two phases of field collections in the current study to assess the sensitivity of the probe and compare results with those obtained via the 16S metabarcoding methodology. Half filters were used and run in triplicate for all samples. Extraction negatives were also included.

4.11 Tissue Collection and Genetic Analyses

4.11.1 Laboratory Methods

4.11.1.1 DNA Extraction

All tissue was collected according to the SoP for '*Tissue Sample Collection and Storage for Mammals*' DBCA (2017g) the SoP for '*Temporary Marking of Mammals, Reptile and Birds*' DBCA (2017h). Tissue samples, stored in 100% ethanol, were maintained at -20°C prior to DNA extraction. DNA was then extracted from 1-2 scales per python using the Qiagen DNeasy Blood & Tissue kit (QIAGEN) following the spin-column protocol for purification of total DNA from animal tissues. This methodology was modified slightly by performing the final elution step twice, using 70 µl and 80 µl of buffer. The resulting purified DNA was then stored at -20°C prior to use.

4.11.1.2 PCR Procedures and Microsatellite Primer Screening

Genomic DNA was mined for microsatellites using a MiSeq Illumina Next Generation Sequencing run (Peter Spencer, Murdoch University, unpublished data). Twenty-five pairs of microsatellite primers were selected from this run, and a further twelve primer pairs were chosen based on Ciavaglia et al. (2019). The total 37 primer pairs were screened for their ability to amplify the extracted POP DNA, by performing a gradient temperature polymerase chain reaction (PCR) to determine the optimal annealing temperature. Thirty primers pairs were subsequently retained owing to their ability to generate a stable PCR product.

M13- tags were added to the 5'- end of all forward primers, following the protocol of Schuelke (2000). To facilitate multiplexing, M13- tags were also added to 6FAM, VIC, NED and PET fluorescent dyes as described by Venkatsen et al. (2007).

PCR amplification was performed on an Eppendorf Thermalcycler using the following procedure: an initial denaturation step of 95°C for 3 minute, followed by 25-35 cycles of 95°C for 30 seconds, annealing at 56-60°C (depending on the locus) for 1 minute, extension at 72°C for 1 minute, then (to facilitate M13- binding) a further 8 cycles of 95°C for 30 seconds, annealing at 53°C and extension at 72°C for 1 minute. Lastly, there was a final extension step of 72°C for 5 minutes. For fragment analysis (FA), 3 µl of the amplified PCR-product was loaded with 15.5 µl Hi-Di formamide (Applied Biosystems) and 0.13 µl Genescan 500 LIZ (Thermo Fisher) internal size standard and run on an Applied Biosystems 3730xl DNA Analyser (ABI, Melbourne). When multiple PCR-reactions (each containing primers with different dye attachments) were loaded in the same well for FA, 5 µl of each PCR reaction was first mixed and 3 µl of the resulting mixture was loaded for FA. Fragments were then scored manually using Geneious V2023.0.3 software.

4.11.2 Population Genetics Analyses

Basic population genetics statistics were generated using R (R core team 2022) software and the excel add-in GenAlEx 6.5 (Peakall and Smousse 2006, 2012). The R package 'PopGenReport' (Adamack and Gruber 2017) was used to assess data quality (percentage of missing data, null alleles), total number of alleles per site and private alleles. We determined the frequency of null alleles per locus using the method of Brookfield (1996). Departures from Hardy-Weinberg equilibrium (HWE) were assessed for each locus and population with the R package 'pegas' (Paradis 2010), using an exact test with 1,000 Monte Carlo permutations and a = 0.05. GenAlEx was used to calculate the number of alleles (N_A), number of effective alleles (N_E) and Information index (I). The R package 'diveRsity' v1.9.90 (Keenan 2013) was used to estimate observed and expected heterozygosities (H_O and H_E), resampled allelic richness (A_R) and the resampled inbreeding coefficient (F_{IS}), with confidence intervals for A_R and F_{IS} calculated using a bootstrap procedure (1,000 randomisations) and a = 0.05. Resampling was used to correct for differences in sample size.

Population genetic structure was assessed in STRUCTURE (volume 2.3.4, Pritchard et al. 2000). The diveRsity package was also used to evaluate genetic differentiation by estimating population

pairwise FST (Weir and Cockerham 1984) and GST (Nei and Chesser 1983) values, with 95% confidence intervals calculated from a bias-corrected bootstrap method (1000 randomisations).

The adegenet 1.3-1 package (Jombart and Ahmed 2011) in R (R core team, 2022) was used to perform both a principal components analysis (PCA) of POP populations and a discriminant analysis of principal components (DAPC) for an additional assessment of genetic structure.

The methods and results were reviewed by Murdoch University Associate Professor Dr. Peter Spencer.

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5.0 Results

5.1 Captures, Radio-tracking and POP movement

5.1.1 Pilbara Olive Python Captures

A total of twenty-eight POP individuals (*Liasis olivaceus barroni*) were detected during the monitoring program either through direct observation or secondary sign (Table 5.1). This comprised of seven individuals at Western Ridge, 15 individuals at Opthalmia Dam and six individuals at Millstream. Twenty-four live POP were hand-captured, while an additional four individuals were identified through genotyping sloughs and deceased remains; two at Western Ridge and two at Ophthalmia Dam (Plate 5.1-2). A final slough collected at Western Ridge (QR46; not depicted in Table 5.1 due to repeat individual) was found to be a genetic match to one of the hand-captured individuals (POP 201). Two individuals at Nankunya in Western Ridge had previously been captured and fitted with microchips at the site; microchip IDs 900193003604512 and 900193003604460 (Biologic 2021).

Two individuals were found deceased via tracking, POP 202 at Western Ridge on 08/09/2022 (Plate 5.1) and POP 205 at Ophthalmia Dam on 10/12/2022 (Plate 5.2). Four POP have had their transmitters replaced in 2023 to maintain and extend battery life; POP 102, 103, and 106 (all Millstream), and POP 203 (Ophthalmia Dam).

Plate 5.1: **QR17; skeletal remains at Western Ridge.**

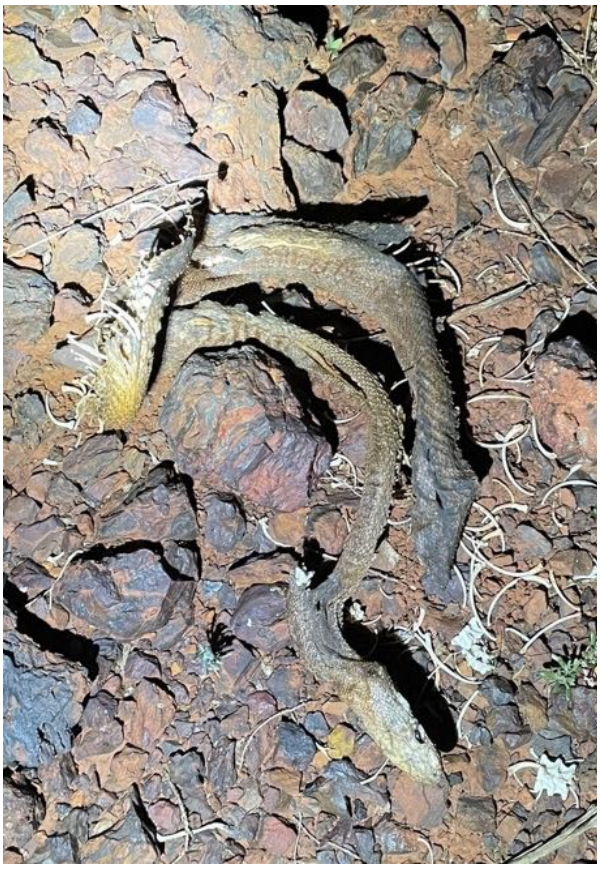

Plate 5.2: **QR44; skeletal remains at Ophthalmia Dam.**

Table 5.1 POP individuals recorded during this monitoring program through either hand-capture or secondary sign (scat, slough, and/or remains).

Scale Clip Code	Capture Date	Latitude	Longitude	Status	Sex	Microchip ID	Tissue Specimen Code	DNA Extraction ID
Western Ridge								
201	16/01/2022	-23.3939608	119.6179277	Hand capture	Female	956000012887025	T20220117.POP07.LIAOLI-01	QR07
202	24/02/2022	-23.3831370	119.6145098	Hand capture	Female	900193003604512	T20220225.POP08.LIAOLI-01	QR08†
211	08/12/2022	-23.3833396	119.6145956	Hand capture	Male	956000016551966	T20221209.POP17.LIAOLI-01	QR32
212	08/12/2022	-23.3831199	119.6145728	Hand capture	Female	956000016551754	T20221209.POP18.LIAOLI-01	QR33
216	26/01/2023	-23.3826718	119.6137541	Hand capture	Female	900193003604460	T20230126.POP23.LIAOLI-01	QR38
-	16/01/2022	-23.3965073	119.6134716	Remains	n/a	n/a	T20220116.POPOPX.Liaoli-01	QR17
-	9/12/2022	-23.3967500	119.6135390	Slough	n/a	n/a	S20221209.LIAOLI-01	QR40
Ophthalmia Dam								
203	26/02/2022	-23.3518780	119.8994080	Hand capture	Female	956000014470808	T20220227.POP09.LIAOLI-01	QR11
204	26/02/2022	-23.3513780	119.8980130	Hand capture	Male	956000012888368	T20220227.POP10.LIAOLI-01	QR10
205	27/02/2022	-23.3117996	119.8674056	Hand capture	Female	956000012885223	T20220228.POP11.LIAOLI-01	QR09†
206	28/02/2022	-23.3520689	119.9006027	Hand capture	Male	956000012886555	T20220302.POP14.LIAOLI-01	QR15
207	28/02/2022	-23.3538016	119.9038105	Hand capture	Female	956000014468617	T20220302.POP13.LIAOLI-01	QR16
208	28/02/2022	-23.3123448	119.8679303	Hand capture	Male	956000012888289	T20220302.POP16.LIAOLI-01	QR12
209	28/02/2022	-23.3518083	119.8993669	Hand capture	Male	956000012885409	T20220302.POP15.LIAOLI-01	QR14
210	28/02/2022	-23.3520261	119.9005030	Hand capture	Female	956000014455686	T20220302.POP12.LIAOLI-01	QR13
213	10/12/2022	-23.353474	119.898286	Hand capture	n/a	n/a	T20221210.POP213.LIAOLI-01	QR34
214	10/12/2022	-23.3530251	119.8976877	Hand capture	Male	956000016561258	T20221210.POP214.LIAOLI-01	QR35
215	23/01/2023	-23.3518794	119.8980479	Hand capture	Female	956000016552530	T20230124.POP21.LIAOLI-01	QR36
217	28/01/2023	-23.3550055	119.8966416	Hand capture	Male	956000016553289	T20230129.POP24.LIAOLI-01	QR39
218	13/05/2023	-23.3428626	119.8893497	Hand capture	Female	956000016551108	956000016551108	QR42
222	23/01/2023	-23.3533856	119.8980851	Hand capture	Female	956000016622324	T20230124.POP222.LIAOLI-01	QR37
-	11/05/2023	-23.3452990	119.8953470	Slough	n/a	n/a	-	QR45
-	13/05/2023	-23.3421161	119.8901623	Remains	n/a	n/a	T20230509.POPDec.LIAOLI-01	QR44

Scale Clip Code	Capture Date	Latitude	Longitude	Status	Sex	Microchip ID	Tissue Specimen Code	DNA Extraction ID
Millstream								
101	11/01/2022	-21.5893991	117.0708251	Hand capture	Male	956000012889132	T20220112.POP01.LIAOLI-01	QR01
102	11/01/2022	-21.5694494	117.0554189	Hand capture	Female	956000012884103	T20220112.POP02.LIAOLI-01	QR03
103	12/01/2022	-21.5699518	117.0543538	Hand capture	Male	956000012887175	T20220113.POP03.LIAOLI-01	QR02
104	12/01/2022	-21.5877477	117.0707235	Hand capture	Female	956000014464906	T20220113.POP04.LIAOLI-01	QR06
105	12/01/2022	-21.5870528	117.0708989	Hand capture	Female	956000014469393	T20220113.POP05.LIAOLI-01	QR05
106	12/01/2022	-21.6070710	117.1059840	Hand capture	Male	956000012887090	T20220114.POP06.LIAOLI-01	QR04

† denotes individuals implanted with radio-transmitters during the survey that have since been found deceased.

5.1.2 Radio-tracking

All POP with VHF transmitters implanted (24 individuals in total) were radio-tracked during subsequent surveys using hand-held yagi antennas, a summary of the location per day and per individual is presented in Figure 5.1. When the signal from a radio-tagged POP was detected, it would be followed until the individual's location was determined. If the location was safely accessible, the animal was tracked to its exact location, with habitat details recorded. If the location was inaccessible, due to either unsafe terrain or restricted tenement access, the snake's location was determined via triangulation from multiple locations. A summary of the movements, behaviour and habitats utilised for each individual POP tracked is presented in Appendix 5.

The number of location events per snake varied between sites. At Ophthalmia Dam, the highest number of location events were recorded, with POP 206 recorded on 10 separate occasions, and several others receiving 8-9 relocation events. Millstream had the fewest locations events, with only 1-3 events per POP, primarily due to site access limitations. At Western Ridge, the number of relocation events was limited, mainly because fewer snakes were captured at the start of the program. Two individuals, POP 201 and POP 216, were each relocated six times. POP 201 was initially captured in January 2022, while POP 216 was captured in February 2023.

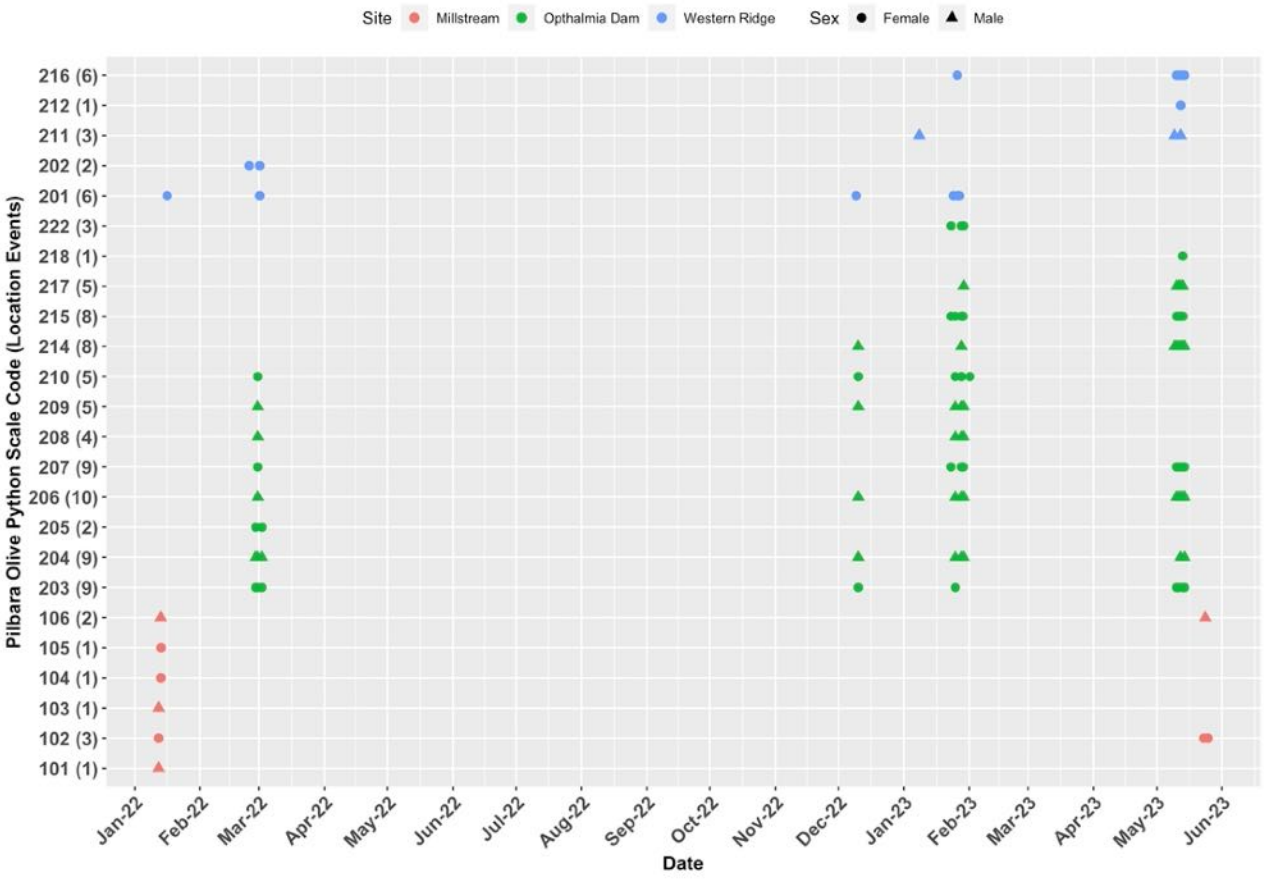

Figure 5.1: Pilbara Olive Python location events over the duration of the monitoring program.

5.1.3 Area of Occurrence

The area occupied by the tracked individual POP was, estimated by its Minimum Convex Polygon (MCP), which represents the minimal convex hull encompassing all recorded locations, providing a conservative estimate of its spatial range. This area, termed the 'area of occurrence', differs from a home range in that it does not account for the frequency and intensity of habitat use; rather, it merely outlines the extent of spatial occupation without factoring variations in activity levels or habitat preferences (Burt 1943). While MCPs are useful for providing a broad overview of an individuals spatial range, they may underestimate the true complexity of habitat utilisation (Burgman and Fox 2003). Calculating MCPs necessitates a minimum of five relocation points, comprising the initial encounter and at least four subsequent relocation events occurring on separate days, as defined in this study. This is comparatively fewer relocation points than is required to estimate a home-range which typically recommends at least 30 independent relocation events over a period of at least six months (Hooten et al. 2017), and in some cases much longer depending on the frequency that an individual traverses its home range (Fleming et al. 2015).

Minimum Convex Polygon were calculated using the package 'adehabitatHR' version 0.4.21 (Calenge 2023) within the software program 'R' version 4.4.0 (R Core Team 2024). The MCPs of 10 POP were calculated which comprised of two individuals from Western Ridge and eight from Ophthalmia Dam (Table 5.2). Due to the insufficient number of relocation events for any individual from Millstream, it was not possible to calculate the Minimum Convex Polygons (MCPs) for these individuals.

The level of stability of the MCPs was calculated for the ten individuals with sufficient relocation events, this comprised two individuals from Western Ridge (Figure 5.2) and eight from Ophthalmia Dam (Figure 5.3). Stability refers to the extent to which the home-range size remains relatively unchanged across a range of home-range levels, broadly referred to as the following:

- Home-range level (x-axis): The percentage of the total area in which the species is found, starting from the most intensively used core areas (lower percentages) to the full extent of the home range (100%); and
- Home-range size (y-axis)**:** This represents the actual size of the area utilised by the population at each corresponding home-range level.

For individuals from both sites, there is a period where the home-range size remains relatively stable as the home-range level increases from 50% to around 80%-90%. This suggests that the individuals are concentrated in a core area that does not expand significantly until higher levels of their area of occurrence are considered. At higher home-range levels (around 80%-90% and above), there is a significant increase in the area of occurrence, indicating that individuals begin to utilise more peripheral, less frequented areas.

The area of occurrence for POP at Western Ridge ranged from 1.8 ha (POP 216) to 16.3 ha (POP 201) (Table 5.2), with POP 216 showing ranges closely associated with the extent of Nankunya (Figure 5.4). Conversely, POP 201's MCP encompassed multiple gorge systems, from Xanadu Gorge to Skeleton Gorge, covering a substantial 16.3 ha expanse (Table 5.2; Figure 5.4). At Ophthalmia Dam, the area of occurrence ranged from 0.49 ha (POP 209) to 33.53 ha (POP 203) (Table 5.2). This variation likely reflects the fewer relocations for POP 209, which were predominantly obtained over two surveys. If a greater number of relocations had been obtained over a longer period, we would expect the area of occurrence to increase.

At Western Ridge, the area of occurrence calculated for POP 212 (1.8 ha) and POP 216 (1.9 ha) were similar, with most relocations centred at Nankunya. In contrast, the third individual, POP 201, exhibited an area of occurrence stretching from Xanadu Gorge to Skeleton Gorge, covering a total area of 16.3 ha (Table 5.2). At Ophthalmia dam, the area of occurrence varied significantly, with the smallest area of occurrence recorded was POP 209, with an area of 0.49 ha, whilst the largest area calculated was POP 203 with 33.53 ha (Table 5.2; Figure 5.5). At Ophthalmia Dam, a

large proportion of individuals were captured within a 2 ha area of Bulrush (*Typha orientalis*) along the shoreline of the dam waterbody. The surrounding habitat consists of low introduced grasses and dead shrubs, facilitating easy detection of individuals. Subsequent relocations indicate that nearly all these individuals move between the rocky incised hills to the north and the dam waterbody (Figure 5.5).

Table 5.2: Pilbara Olive Python tracking duration, number of relocations and area of occurrence.

Scale ID	Age and Sex	First Location Event	Latest Location Event	Tracking Duration (months)	Relocation Events*	Area of Occurrence (ha)	Habitat utilised
Western Ridge							
POP 201	Sub-adult female	16/01/2022	27/01/2023	12.5	5	16.3	Waterbody (pools), gorge/gully, hillcrest/ hillslope, stony plains, mulga woodland and drainage area/ floodplain
POP 202	Adult female	24/02/2022	08/09/2022	6.5	1	-	
POP 211	Adult male	08/12/2022	12/05/2023	6	2	-	
POP 212	Adult female	08/12/2022	-	-	-	-	
POP 216	Adult female	26/01/2023	14/05/2023	3.5	5	1.9	
Ophthalmia Dam							
POP 203	Adult female	26/02/2022	14/05/2023	14.5	8	33.53	Waterbody (dam), Typa reedbed, hillcrest/ hillslope, stony plains, mulga woodland and drainage area/ floodplain
POP 204	Adult male	26/02/2022	14/05/2023	14.5	8	16.48	
POP 205	Juvenile male	27/02/2022	10/12/2022	9.5	2	-	
POP 206	Adult male	28/02/2022	14/05/2023	14.5	9	31.53	
POP 207	Adult female	28/02/2022	14/05/2023	14.5	8	13.65	
POP 208	Adult male	28/02/2022	29/01/2023	11	3	-	
POP 209	Juvenile male	28/02/2022	29/01/2023	11	4	0.49	
POP 210	Juvenile unsexed	28/02/2022	29/01/2023	11	4	1.92	
POP 214	Adult male	10/12/2022	14/05/2023	5	7	8.11	
POP 215	Adult male	23/01/2023	13/05/2023	3.5	7	0.98	
POP 217	Adult male	28/01/2023	14/05/2023	3.5	4	NA	
POP 218	Adult female	13/05/2023	-	-	-	-	
POP 222	Juvenile female	23/01/2023	29/01/2023	1	2	-	
Millstream							
POP 101	Adult male	11/01/2022	26/10/2022	9.5	0	-	Waterbody (pools), hillcrest/hillslope, Typha reedbed, drainage line (creek)
POP 102	Sub-adult female	11/01/2022	26/05/2023	16.5	2	-	
POP 103	Sub-adult male	12/01/2022	25/05/2023	16.5	0	-	
POP 104	Sub-adult female	12/01/2022	26/05/2022	5.5	0	-	
POP 105	Juvenile male	12/01/2022	26/10/2022	9	0	-	
POP 106	Sub-adult male	12/01/2022	24/05/2023	16.5	1	-	

*Relocation Events excludes the initial capture.

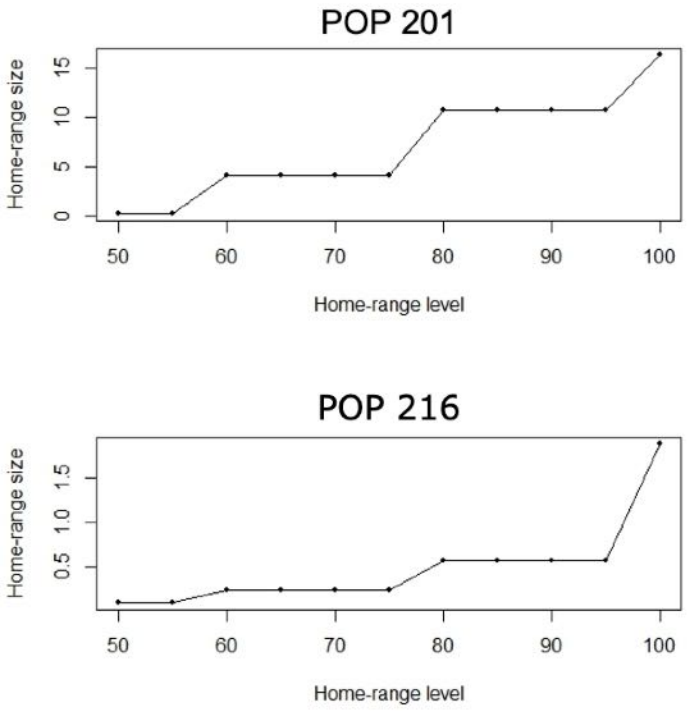

Figure 5.2: Area of occurrence stability of Pilbara Olive Pythons tracked at Western Ridge based on percentage of relocation event.

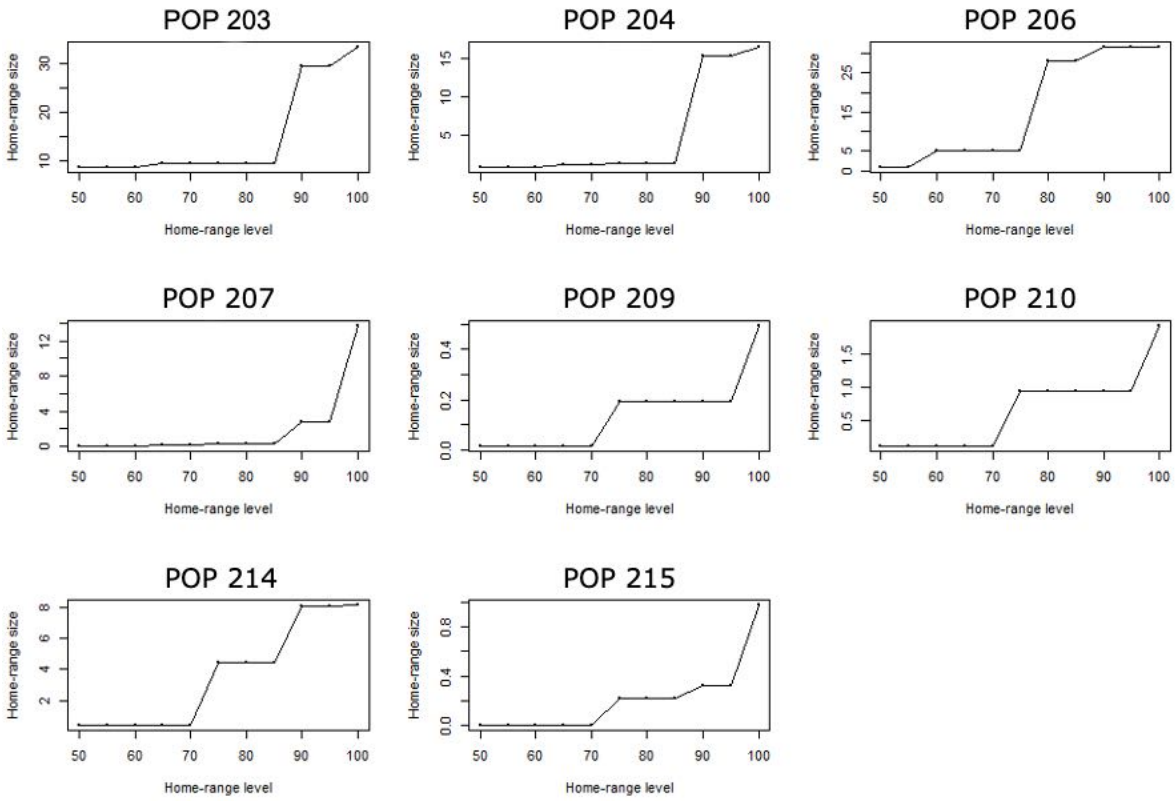

Figure 5.3: Area of occurrence stability of Pilbara Olive Pythons tracked at Ophthalmia Dam based on percentage of relocation event.

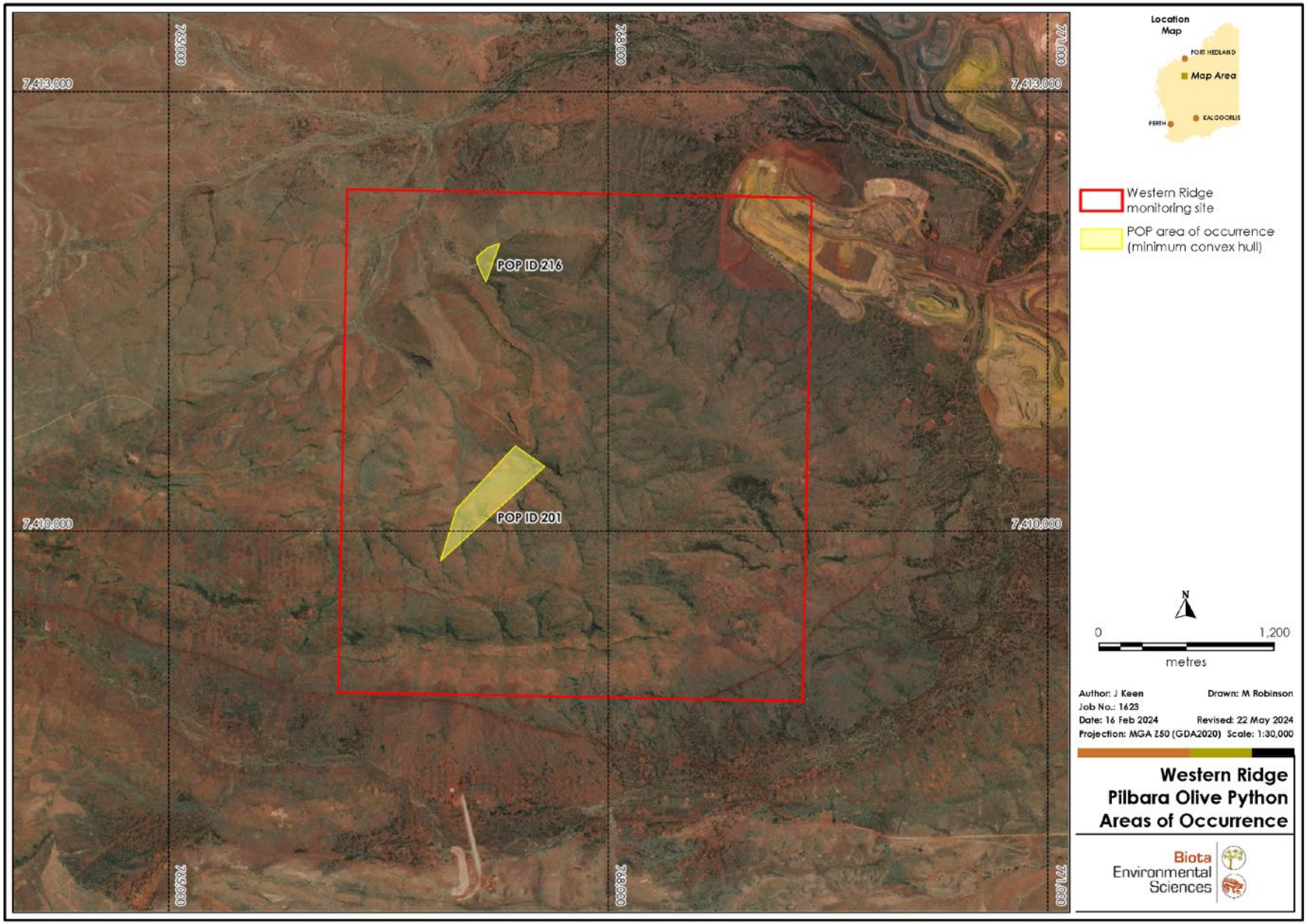

Figure 5.4: Minimum convex polygons based on the individual relocation events of tracked Pilbara Olive Pythons at Western Ridge.

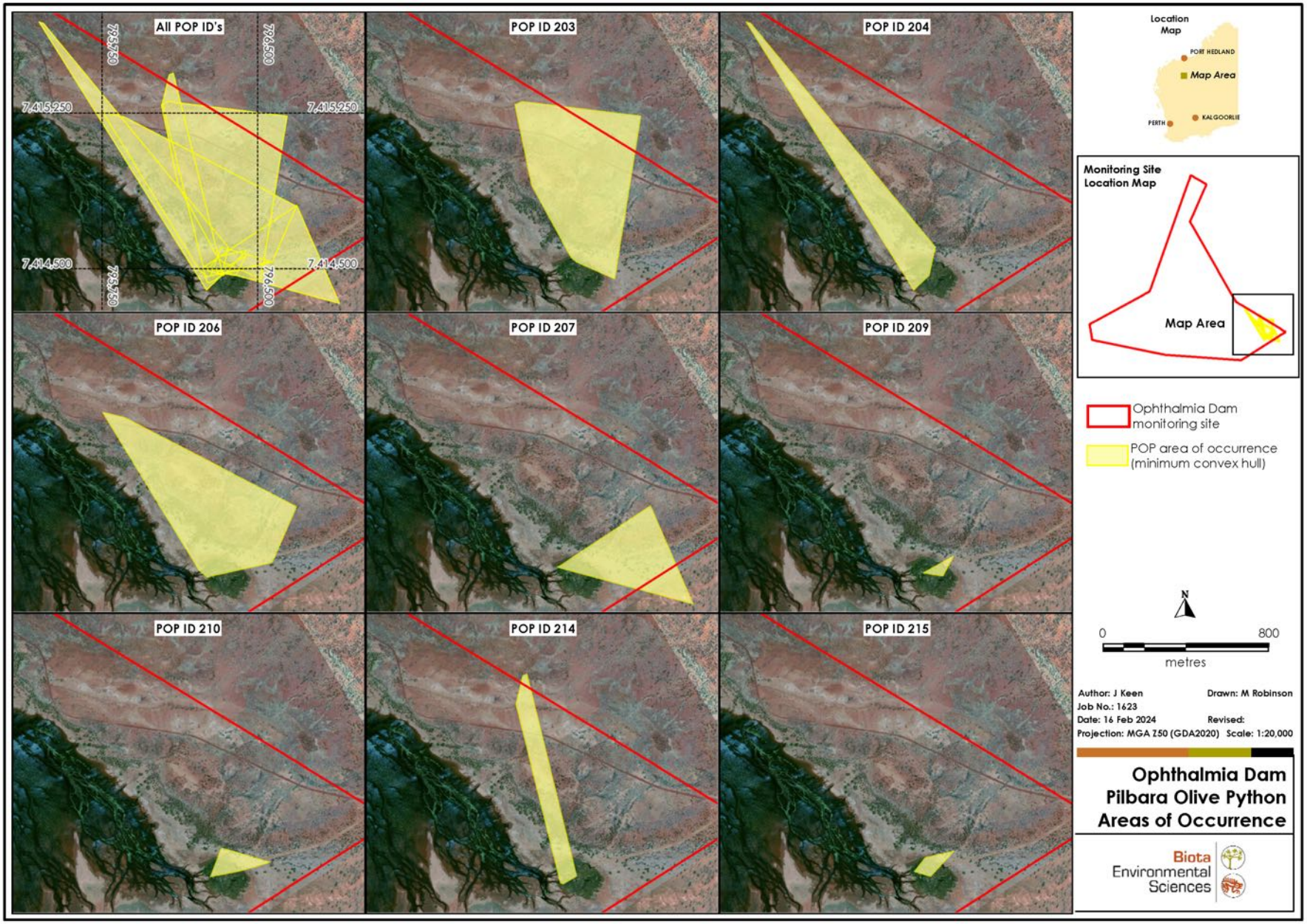

Figure 5.5: Minimum convex polygons based on the individual relocation events of tracked Pilbara Olive Pythons at Ophthalmia Dam.

5.2 Motion Cameras

No POP were observed on motion cameras deployed at Western Ridge during the 245 camera-nights of effort expended during the monitoring period. This was expected, given that standard motion cameras do not reliably detect reptiles (see Appendix 1). Motion cameras did detect two macropod species, both of which are suitable prey for large POP; Rothschild's Rock-wallaby (*Petrogale rothschildi*; Plate 5.3, Plate 5.5, Plate 5.6) and Euro (*Osphranter robustus*; Plate 5.4).

Euros are common at Western Ridge: they have previously been recorded (see Plate 3.3 in Biologic 2021), their scats were commonly observed during the targeted searches and radio-tracking, and they were observed on all motion cameras except POP02MC. However, from the available data these are the first records of Rothschild's Rock-wallaby at Western Ridge (cameras POP01MC and POP04MC). Dingoes (*Canis familiaris dingo/familiaris*), a potential predator of POP, were recorded on both cameras in Nankunya gorge (POP05MC and POP06MC).

Plate 5.3. Rothschild's Rock-wallaby (*Petrogale rothschildi*) at POP04MC.

Plate 5.4. Euro (*Osphranter robustus*) at POP04MC.

Plate 5.5. Adult and juvenile Rothschild's Rock-wallabies (*Petrogale rothschildi*) at POP01MC.

Plate 5.6. Adult and juvenile Rothschild's Rock-wallabies (*Petrogale rothschildi*) at POP01MC.

Plate 5.7. Dingo at POP05MC.

Plate 5.8. Dingo at POP06MC.

5.3 Population Genetics

Full results of the population genetics study are presented in Appendix 7, with a summary presented here.

Individuals were successfully identified from all tissue and slough samples. All 30 microsatellite loci successfully amplified in each of the 29 individuals used in this study, with a missing data rate of only 0.03%. Eleven of the 30 loci remain monomorphic (holding only one allele) in all individuals and so were removed from further genetic analyses, while all 19 polymorphic loci were retained. No null alleles were detected. Three loci showed significant deviations from HWE in the Ophthalmia Dam subpopulation, however, as they were in equilibrium in the other two subpopulations they were not excluded from the analysis.

All within-population descriptive diversity measures identified the subpopulation at Ophthalmia Dam as more genetically diverse than either Millstream or Western Ridge, but only by a marginal amount (not statistically significant). Ophthalmia Dam held the highest number of alleles (mean N_a= 3.26 ± SE 0.43), effective alleles (mean N_e = 2.44 ± SE 0.29) and private alleles (P_a = 11), as well as the highest values for allelic richness (mean A_r = 2.68 (95% CI 2.37 – 3.00)), observed and unbiased expected heterozygosity (mean H_o = 0.50 ± SE 0.07, uH_e = 0.49 ± SE 0.06), and information index (mean I = 0.85 ± SE 0.20).

Slightly lower but comparable levels of diversity were observed at both Millstream (mean N_a = 2.74 ± SE 0.30, N_e = 2.00 ± SE 0.22, P_a = 7, A_R = 2.45 (95% CI 2.10 – 2.74), H_o = 0.47 ± SE 0.07, u_{He} = 0.45 ± SE 0.06, I = 0.71 ± SE 0.10) and Western Ridge (mean N_a = 2.79 ± SE 0.28, N_e = 1.90 ± SE 0.19, P_a= 5, A_R = 2.37 (95% CI 1.95 – 2.68), H_o = 0.39 ± SE 0.06, u_{He} = 0.41 ± SE 0.06, I = 0.67 ± SE 0.10). Overlapping error bars for allelic richness indicate there are no statistically significant differences in this measure between any of the sampled subpopulations, supporting the inference that observed levels of genetic diversity are broadly similar between the three sites. While similar, Western Ridge and Ophthalmia Dam were found to be more genetically comparable to each other than with Millstream.

The inbreeding coefficient (FIS) was low in all three subpopulations, with mean FIS ranging from -0.15 (95% CI -0.40 - -0.11, Millstream) to -0.03 (95% CI -0.16 - 0.04, Ophthalmia Dam). Negative values such as this indicate that inbreeding within the subpopulations is rare and genetic diversity is high.

Genetic diversity was highest in the Ophthalmia Dam POP and lowest at Western Ridge, however this may be due to sampling effect, as each of these populations respectively contain the highest and lowest number of sampled individuals.

The greatest genetic differentiation observed, as indicated by FST was between Millstream and all other populations. This result is supported by the STRUCTURE analysis.

5.4 Genetic Relatedness

The genetic relatedness study was used to identify and characterise the level of gene flow that occurs between and within each of the three study sites. Full results of the genetic relatedness study please see Appendix 8, with a summary presented here.

When data from all POP individuals sampled from all monitoring sites during this study were pooled into a single population for analysis purposes, genetic relatedness (*Rxy*) ranged from -0.738 to 0.731, with an average of -0.082 (+/- SD 0.251) indicating that the majority of sampled individuals are unrelated to one another. When subpopulations from each of the monitoring survey sites (Western Ridge, Ophthalmia Dam and Millstream) were analysed separately, mean relatedness values showed a similar result and retain the signature of no genetic relationship.

The lowest mean *Rxy* (genetic relatedness) was found at Western Ridge, with an average of -0.142 (+/- SD 0.334). This subpopulation also has the largest range, with values from -0.817 to 0.550. *Rxy* of the Millstream subpopulation ranged from -0.659 to 0.627, with an average of -0.091 (+/- SD 0.319). Average relatedness is marginally higher (but not statistically significant) in the Ophthalmia Dam subpopulation, where *Rxy* ranges from -0.661 to 0.488 and averages -0.030 (+/- SD 0.239). These results make sense when viewed alongside the negative FIS values which indicate large, diverse and outbred populations (Appendix 7).

Relatedness between individuals was separately assessed against both stringent and relaxed datasets. First-order relationships between individuals (e.g. parent-offspring or full sibling) were discovered at all sites in both the stringent and the relaxed analyses. The details of these relationships are explained in further detail below.

When the three subpopulations were analysed in isolation using the stringent dataset, Western Ridge was found to support an intermediate percentage of related individuals, relative to the other two subpopulations. Ten percent (two out of 21 dyads) of all dyads (pair of individuals) analysed from the Western Ridge subpopulation are related to one another, with 5% of dyads highly related accordant to the first-degree level (one parent-offspring dyad) and another 5% moderately related to the level of second-degree. Millstream contains the largest percentage of related individuals, with first-degree relationships (one parent-offspring, one full-sibling dyad) detected in 13% (2 out of 15 dyads) of dyads analysed. Ophthalmia Dam displayed a slightly lower percentage (8%, 10 out of 120 dyads) of related pairs than that found at Western Ridge and Millstream.

When the analyses were relaxed, a significantly higher number of second-order relationships were uncovered at all sites. Second-order relationships include extended familial relationships such as aunts, uncles, nephews, nieces, grandparents, grandchildren, half-siblings and double-cousins. One additional second-order relationship was identified at Millstream, 16 at Ophthalmia Dam and three at Western Ridge, increasing the overall percentage of related dyads at each subpopulation to 20%, 24% and
24%, respectively.

Genetically important individuals may be regarded as those who share genes with a large proportion of the population. In the relaxed analysis, POP 210, a young female python at Ophthalmia Dam, was found to have a total of eight known relationships (two first-degree, six second-degree); the highest number of this study. At Western Ridge, POP 202 and/or QR17 (a python known only from musculo-skeletal remains) were identified in all five of the detected relationships (POP 202 with three second-degree relationships, and QR17 with one first-degree and two second-degree relationships). At Millstream, POP 105 with two identified relationships (one first-degree and one second-degree), is considered the most genetically important individual; all other related specimens sampled from this monitoring study site are linked to only one other individual.

While genetic dispersal between the monitoring sites was rare, the greatest proportion of between-site genetic relatedness connections exist between the most geographically proximate sites Western Ridge and Ophthalmia Dam (eight of 406 dyads, 2%), followed by Western Ridge and Millstream (six of 406 dyads, 1%). Only one relationship was detected between Ophthalmia Dam and Millstream, suggesting that there may be some landscape resistance precluding dispersal between these sites, or sampling effect has impaired the identification of more relationships.

5.5 eDNA

eDNA samples collected in Phases 1, 2, and 4 were analysed by 16S metabarcoding only. During Phase 1, 28 filters were analysed from 12 sites; 18 filters from Millstream and 10 from Western Ridge. Of these, one filter at each site tested positive for POP. In Phase 2 a total of 46 filters were analysed from 16 sites, 28 filters were analysed from Western Ridge, with nine testing positive for POP. Whilst 22 filters from Millstream were analysed returning only one positive result. In Phase 4, 38 filters were analysed from across Western Ridge (n=15 from 3 sites) and Ophthalmia Dam (n=21 from 7 sites), of which five tested positive for POP; three at Western Ridge and two at Ophthalmia Dam. A further 15 filters from 5 sites at Millstream were analysed from phase 6, none of which tested positive for the presence of POP. Full eDNA results (eDNA Frontiers reports) are presented in Appendix 9.

Across Phases 5 and 6, 42 eDNA filter samples were analysed using both 16S metabarcoding and the newly developed species-specific probe-based qPCR. Across the 27 filters tested (from both Western Ridge and Ophthalmia Dam), both eDNA tests agreed on negative results on 18 filters, agreed on positive results for two filters, and did not match on the remaining seven (three of which metabarcoding determined were positive, while the probe returned negative, and four of which the probe determined were positive, while the metabarcoding returned negative).

Results were much clearer during the Phase 6 Millstream trial, where 15 filters were directly compared. For all filters, the metabarcoding technique did not detect the presence of POP, while the probe returned six positive results. This likely reflects the higher sensitivity of the probe relative to the metabarcoding method; particularly as Millstream sampling sites were typically characterised by flowing water, which is likely to affect the concentration, integrity and persistence of eDNA.

6.0 Discussion

The total survey effort across all phases and all sites was 295.1 hours, comprised of 123.2 hours at Western Ridge, 104.3 hours at Ophthalmia Dam and 67.65 hours at Millstream. The survey effort yielded a total of 28 POP individuals (24 of which were live), comprising seven individuals at Western Ridge, sixteen at Ophthalmia Dam and six at Millstream.

6.1 Monitoring Approaches: Learnings and Considerations

6.1.1 Detectability: Insights from Radio-tracking

Monitoring programs developed for POP are often stymied by low detectability and re-detectability of individuals, attributed to the species' cryptic nature and the existing knowledge gaps surrounding their ecology. Low detection rates significantly hinders implementing statistically-robust monitoring techniques, such as distance sampling and spatial mark-recapture (Clement et al. 2017). As such, the prevailing methods for monitoring POP for development projects throughout the Pilbara have primarily been limited to targeted searches in suitable habitat and eDNA sampling at rock pools, usually carried out on a siloed site-by-site basis. This program is the first instance in which radio-telemetry was implemented to account for low detectability.

The aim of implanting individual POP with VHF transmitters was to increase the likelihood of their redetection during subsequent surveys. Over the duration of the program, it was identified that even with VHF transmitters implanted, the capacity for an observer to locate pythons could be influenced by their location or usage of microhabitat, as signals can be impeded by water and terrain. There were several individuals which could not be located between survey phases, and it is unknown whether this was due to the animal moving beyond the range detectable by the telemetry receiver, or if the individual was utilising refugia that prevented the signal from being detected. After individuals were initially located and captured, none of those with VHF transmitters at Western Ridge were incidentally redetected during targeted searches; all required the use of radio-telemetry for relocation. This highlights the importance of improving redetection likelihood, especially for cryptic species like POP, when attempting to determine their movements and spatial utilisation or for monitoring.

At Western Ridge there were two POP captured that had been microchipped during surveys prior to the commencement of the current program. The first instance was a female (POP 202), initially captured by Biologic on the 15th of March 2020 (Biologic 2021). This individual was recaptured by Biota on the 24th of February 2022, approximately 23 months later and 160 metres southeast of the initial capture point, and within the same gorge feature. The second instance involved an adult female (POP 216), first captured by Biologic on August 27, 2020 (Biologic 2021). This individual was recaptured by Biota on the 26th of January 2023, approximately 29 months after its initial capture.

The re-detection of individuals was highest at the Typha patch on the eastern side of the Ophthalmia Dam study area, where the open muddy landscape interspersed with scattered stag trees and eucalypts facilitated POP detection. Even so, only two snakes were re-detected without radio-tracking at this site. No individuals were re-detected without radio-tracking at Millstream. Despite the advantage of using radio telemetry, locating POP in the site's dense riparian vegetation proved challenging.

Whilst ground-based radio-tracking far outperformed visual searches for POP re-detection, it was identified that dense ironstone ridges and hills could significantly hinder VHF signal strength. In some instances, the largest transmitters (AI-T2) were detected from approximately 3 km away when uninterrupted by ridges or hills. However, signal strength could be significantly reduced or completely lost if a python moved deep into an ironstone crevice. To overcome this, future VHF monitoring should consider allowing several days of tracking per attempt, with attempts spaced

throughout the year. Pythons should not be assumed to be absent from a site if no signal is detected on any one survey. In future, radio-tracking with drones may relocate pythons faster and GPS transmitters may allow for much greater data collection upon relocation (noting that GPS-tracking technology is still in its infancy for pythons).

The refuges utilised by the radio-tracked POP in this study meant animals were typically well hidden; beneath rocks and rockpiles with small entrances, deep within crevices, or within thick vegetation. In nearly all instances, the POP could not have been detected by visual searches, and would have remained unaccounted for, even if they were within a surveyed area. The radio-tracked POP also spent significant portions of their time away from water bodies, which are the areas most likely to be visited for both targeted searches and eDNA sampling. This included recording POP in habitats typically not expected for the species, such as open spinifex plains and mulga woodlands. Nocturnal searches targeting POP do not typically extend to these habitats, which also often lack standing waterbodies froom which to collect water samples for eDNA.

The tracked POP also moved significant distances: POP 205 (Ophthalmia Dam) moved approximately 750 m in two nights, while another (POP 201; Western Ridge) ranged throughout several dry ridges, gorges and gullies over the course of the study. This latter snake, in the centre of the Western Ridge site, also had no access to a regular water source and thus would not have been detected through eDNA. These findings align with previous POP research, which found POP undertook significant movements in short spaces of time and had home ranges of up to 4.7 km^2 on the Burrup Peninsula (Pearson et al. 2004)

The above observations indicate that any short duration targeted search effort, even if supplemented by eDNA sampling, would likely underestimate the true abundance and distribution of POP at a specific site. This underestimation is compounded by factors such as the scale of the study area, variable detection rate (i.e. season, time of day etc.), availability of habitats, population size, and logistical constraints. This study underscores the advantages of integrating multiple monitoring methodologies into a cohesive program. Radio tracking individuals increased redetection of individuals which greatly improved the understanding of how POP utilised the specific study areas, namely the habitats within which they are most readily redetected, refuge sites utilised, and overall area of occurrence. Sampling waterbodies for eDNA allows for a relatively inexpensive and logistically simple methodology for detecting POP that can be conducted at a large scale, however it is still limited in its capacity to provide important information about populations and their changes.

6.1.2 Movement Data and Habitat Usage

The area of occurrence was estimated for ten individual POP between December 2021 and May 2023. The duration of tracking for the longest monitored POPs was approximately 14.5 months (POP 203, 204, 206 and 207), which allowed for relocation attempts to be obtained over multiple seasons. Findings indicated that the relationship between relocation events and area of occurrence varied among different, populations and locations, highlighting the importance of considering individual variability in habitat use and movement patterns. It should be noted that convex hulls have the potential to overestimate the home range of individuals given that the area within the convex may not be utilised by the individual evenly or at all (Burgman and Fox 2003), for example when an individual is traversing over stony hills between foraging areas in adjacent rocky gorges.

Studies on the movement patterns of POP have documented individuals undergoing brumation, a form of hibernation, during the cooler winter months, seeking refuge in caves and rock crevices away from water sources (Pearson 2003). Conversely, during the warmer summer months, POP have been observed to exhibit extensive movements, often staying near water sources and outcrops (Swan, 2007). Similarly, data collected during the current study revealed that POP movements were more localised and limited during the cooler months and prior to rainfall in summer. This pattern was characterised by greater movements, followed by periods of minimal to no movement during the cooler months. Similar to existing data from Millstream (D.

Pearson pers comm. 2023), the Western Ridge POP exhibited a pattern of moving away from the gorge gulley riverine/spring habitat into the elevated rocky gorge areas to brumate. POP movement, albeit minimal during the cooler months, remained in the rocky refugia. At Ophthalmia Dam, movement data showed a similar pattern, but the distances travelled to transition into rocky refugial habitats were notably greater than previously recorded or expected for POP. Moreover, in many cases, POP had to traverse stony flats and mulga habitats to access suitable structures within incised hills used as brumation sites.

Whilst there have been POP radio-tracked at Pannawonica, Tom Price, Millstream and the Burrup Peninsula in the Pilbara, the only data attainable for comparison is that on five POP individuals from the Pistol Ranges on the Burrup Penninsula (Pearson, 2006). These were radio-tracked for up to three years, with regular tracking events (weekly) to estimate home-range sizes via kernel-density estimates (Pearson 2006). Four of the five individuals were female (2 juvenile, 2 adult) and one was an adult male. According to the home-range estimates they varied from 87.76 to 449.26 ha, an area far exceeding that documented for other Australian pythons (Pearson 2006). In contrast, during the current study, the largest area of occurrence recorded at each site (excluding Millstream) was 16.3 ha at Western Ridge and 33.5 ha at Ophthalmia Dam.

Radio-telemetry across all monitoring sites found POP using hillcrest/hillslope habitat (i.e. hills and ridgelines) as shelter sites, with a notable preference for northwest faces in cooler months (e.g. POP 201, 211 and 216 at Western Ridge, POP 103 at Millstream, and POP 203, 204, 206, 207 and 214 at Ophthalmia Dam). These results show the species utilises these habitat types more than previously thought and suggest it could be considered primary habitat. Hillcrest/hillslope habitat comprises 69.5% of the Western Ridge monitoring site. Additionally, POP were also tracked to stony plains, mulga woodland, and drainage area/floodplain habitats. Data from this study shows that each of these habitats should be considered "secondary" habitat for POP; habitats that individuals are likely to transit through whilst moving between primary habitats of hills, ridges, rock piles and water bodies; with drainage areas and floodplains likely to also represent primary habitat if they are regularly inundated and with access to rocky shelter sites within several hundred metres. These observations largely align with the POP tracking results of Dr. David Pearson (Pearson et al. 2004; D. Pearson unpublished data), which indicate that a larger portion of the Pilbara represent critical or supporting habitats than is currently recognised.

Areas of occurrence estimates offer initial insight into space use by POP, but are likely limited in terms of accuracy by relatively few redetections, compounded by varying intervals between redetections (days to months), seasonality of detections (cool months versus wet months) and when tracking commenced (early or late in the program). Minimum convex polygon estimates potentially bias towards larger estimates of area of occurrence due to outlier relocation events (Burgman and Fox 2003). This can be overcome using kernel density estimation to identify areas within their home ranges are primarily utilised, however this recommends at least 30 independent relocation events over a period of at least six months (Hooten et al. 2017). The independence of relocation events can be further confounded by the time it takes an animal to traverse the entirety of its home-range.

The capacity to investigate trends, such as seasonal movement patterns and demographic disparities, is somewhat constrained by the limited number of relocation events. As the number of relocations increases in the current study, it becomes feasible to explore the factors influencing spatial utilisation more comprehensively.

6.1.3 Insights from Genetic Diversity and Relatedness

The genetic relatedness investigations completed here offer an additional element to POP monitoring which can be explored further with additional specimens in the future. The lack of inbreeding detected at any site in this study, and the presence of an individual at Western Ridge that genetically aligned with those at Ophthalmia Dam, reinforces the findings of the radio-telemetry work: sub-populations of POP are not restricted to permanent water bodies or gorges, and appear to be genetically well dispersed throughout the wider landscape. This may be mediated by significant movement of individuals (at either the juvenile or adult stage) from

hotspots throughout the landscape, or by sub-populations being larger and more interconnected than has previously been recognised, or both.

The low level of inbreeding, moderate-high genetic diversity and large percentage of unrelated pairs of individuals detected – despite the relatively isolated nature of the study sites – suggests population boundaries are larger than each of the monitored sites, and that individuals are dispersing into each site from outside the monitored area. Another possibility for the large number of unrelated individuals is small sample sizes, whereby an insufficient percentage of the population has been sampled to adequately characterise it. Continued sampling within sites, and broader sampling outside of, and between, the currently sampled sub-populations will help determine the effective dispersal distances of individuals and may inform how both natural and anthropogenic landscape features affect gene flow within populations throughout the species' range.

Interpretation of the genetic relatedness of POP with respect to sex and age structure does not reveal any compelling trends. However, relatedness tends to be more prevalent between adults and juveniles, rather than between adults. This is supportive of dispersal events contributing to the low inbreeding population health results. Each monitoring site appears to encompass genetically diverse individuals, rather than closely related individuals. Currently, no obvious patterns exist that would suggest one sex is dispersing rather than the other, nor that this dispersal exists at a particular age-structure (e.g. if male POP were to disperse when they reach sexual maturity). However, an increased sample size may reveal more insights into the patterns of POP dispersal.

6.1.4 Role of Motion Cameras

Standard motion cameras should not be considered a reliable method for detecting pythons. As noted in Section Appendix 1, they are likely to miss potential detections due to their design. The majority of camera models operate with a passive infra-red sensor designed to trigger with a combination of heat and motion and they are therefore often unreliable for detecting reptiles. For example, New motion camera designs, including after-market alterations to existing common models, are being developed to better target reptiles (Hobbs and Brehme 2017), but were not used in this program.

Additionally, the efficacy of motion cameras often relies on an attractant to improve detection. which for snake species presents ethical and logistical constraints using live-prey as attractants. For instance, a monitoring program for the Brown Tree Snake (*Boiga irregularis*) demonstrated this approach by using cameras along with specially designed cages that housed live mice to lure the snakes into the camera's range (Amburgey et al. 2021). This method proved effective for detecting the snakes but highlighted the challenges of balancing detection efficacy with ethical considerations. Pythons may also be more or less likely to be recoded by cameras depending on factors such as the availability of prey which can vary seasonally (Amburgey et al. 2021). . This variability adds another layer of complexity to interpreting the data collected from such studies, as it can be difficult to determine whether a lack of detections is due to the absence of snakes or the inadequacy of the attractant.

6.1.5 Role of eDNA

While eDNA is becoming a popular method for detecting and monitoring POP (Mousavi-Derazmahalleh et al. 2023), it carries significant limitations, several of which have been discussed in Section 6.1.1. These limitations include that null results do not demonstrate POP absence, positive records are incapable of contributing to abundance or density estimates, and that POP eDNA can only currently be sampled at water bodies. Furthermore, these water bodies are not necessarily conducive to the retention of eDNA or its sampling. Common conditions that may inhibit successful eDNA collection include warm or flowing water (which degrade and disperse eDNA) and high silt or algal loads (which inhibit successful filtering of samples).

eDNA will continue to play a role in POP monitoring across its range, and positive records can assist with the DSEWPaC (2008) Approved Conservation Advice goal of locating additional populations/occurrences of POP. However, as it cannot provide information on individuals, age classes, spatial use, movements, abundance, or density, and cannot track changes in any of these factors over time, it can provide little further information to assist land managers in creating site or regional management plans for POP, or with subsequent monitoring and evaluation of the success of such management plans.

eDNA should be seen as a tool which can be beneficial in a limited range of applications and has its primary value in its potential to relatively rapidly confirm the species' presence in the event positive detections are made. This is particularly the case with the newly developed probe-based qPCR for POP, which provides a more sensitive and rapid assay than the metabarcoding method utilised to date.

6.1.6 Conservation Advice Research Priorities

This monitoring program has contributed towards all three of the Research Priorities for the species identified in the Approved Conservation Advice (DEWHA 2008):

- **Priority 1**: *Design and implement a monitoring program*;

A robust ecological and genetic monitoring program has been designed and implemented across three independent POP sub-populations, including expanding the Millstream POP monitoring program being conducted by DBCA's Dr. David Pearson.

- **Priority 2**: *More precisely assess population size, distribution, ecological requirements and the relative impact of threatening processes*;

This monitoring program has provided further detail on the ecological requirements of the species, through radio-tracking, observation and analyses of genetic relatedness within and between sites. This information can be used to inform an assessment of the relative impact of threatening processes on the species.

- **Priority 3**: *Undertake survey work in suitable habitat and potential habitat to locate any additional populations/occurrences*.

The program to date has broadened the distribution of POP records at both Western Ridge and Ophthalmia Dam, and broadened the range of habitats within which they are known to utilise.

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Appendix 1

Challenges with current monitoring approaches

Background

Since the release of the Approved Conservation Advice, significant effort has been placed into undertaking survey work for POP across the Pilbara, particularly by the mining and development sector, and many mine sites now incorporate POP monitoring into their environmental works. However, POP has proven to be a challenging species for sites to effectively monitor in a statistically robust framework due to the difficulty in obtaining sufficient individuals to monitor trends relative to putative impacts.

Therefore, most programs have resorted to finding evidence of persistence (or occupancy) in impacted areas between annual monitoring events. This approach has purportedly been improved upon by the relatively recent adoption of eDNA techniques, but there are important potential limitations with this approach that is detailed in Section 0.

Likelihood of Detection

It is important to acknowledge and evaluate the constraints linked with each monitoring methodology to present a comprehensive understanding of the detailed program, whether these pertain to detection likelihood or the ability to gauge abundance. Doing so allows these limitations to be addressed when developing an effective monitoring program.

Targeted Searches

The key limitation of a simple presence / absence approach using data from targeted searches (hereafter occupancy) in a monitoring framework, is that it does not account for changes in abundance and some impacts may therefore go undetected. Furthermore, none of the POP monitoring studies account for the probability of detection (i.e. given that a site is occupied by a POP what is the probability that the POP will be detected?).

A naïve occupancy approach, that does not take into consideration the probability of detection, is extremely limited as null results cannot confidently be ascribed to true absence. The positive records also then tend to skew such data sets, especially in the context of species distribution models. Failing to account for changes in abundance or providing any estimate of population size also means that three of the IUCN Criteria cannot be evaluated, and hence an informed decision about the conservation rank of POP cannot be made.

Environmental DNA

Environmental DNA (eDNA) approaches rely on the collection and assaying of environmental samples that contain residual DNA that has been shed into the environment from target species. The technique has become an increasingly valuable method for aquatic ecosystems due to the ability to test larger spatial extents for target species using non-invasive methods. There is a rapidly growing number of studies that have successfully used eDNA methods to indirectly detect the presence of aquatic species in marine, estuarine and freshwater systems (Lodge et al. 2012, Minamoto et al. 2012, Thomsen et al. 2012), in addition to groundwater systems (Biota and Helix 2014, 2023). This has also led to the use of eDNA detection methods in other animals including reptiles (Adams et al., 2019), more specifically pythons (Piaggio et al., 2019; Mousavi-Derazmahalleh et al., 2023).

eDNA is an emerging method for detecting the presence of POP, and one that requires less effort than targeted searches. The approach involves collection of water samples from rock pools in which POP have potentially waited to ambush prey items. However, using eDNA to detect terrestrial reptiles is generally less efficient than for mammals, as reptiles are not continually shedding epithelial cells in the same way that mammals do. Rather, avenues for

POP DNA to be found in the water, would be restricted to activities such as urination or defecation whilst in the water, as well as tongue flicking or nasal bubble blowing. It is also known that POP can spend long periods of time away from rock pools (Pearson et al. 2004), meaning the technique is potentially further constrained. More fundamentally, the approach still only provides evidence of presence and without an understanding of the probability of detection (as estimated in an occupancy modelling framework), null records cannot be ascribed to true absence.

An occupancy approach using eDNA cannot provide an estimate of abundance. Arguably, it does not provide any substantive information that would be useful for assessing or re-assessing the conservation ranking of the species. Area of occupancy (a criterion used in IUCN assessments) can be estimated from occurrence records alone, but such records are of limited value if the probability of detection and null records are excluded.

Motion Cameras

Motion cameras are another method gaining popularity for monitoring POP. They may afford a better alternative to determining occupancy than eDNA as they operate over much longer time-periods, and can be placed in dry habitats, such as along ridges, rockpiles, and gorges.

However, most operate on a passive infrared (PIR) sensor, designed to detect mammals based on a combination of heat and motion, with an animal typically needing to be 2.7°C warmer than its surrounding environment and moving across the PIR sensor's field of view to trigger a detection (Hobbs and Brehme 2017). As such, they are often unreliable for detecting reptiles, and indeed most POP detections we have recorded on motion cameras occur when a warm-blooded animal has triggered the camera, and the POP is simply present in the background.

Appendix 2

DBCA License

GOVERNMENT OF WESTERN AUSTRALIA

Department of **Biodiversity, Conservation and Attractions**

AUTHORISATION TO TAKE OR DISTURB THREATENED SPECIES

Section 40 of the Biodiversity Conservation Act 2016

AUTHORISATION DETAILS

Authorisation type: Fauna

Authorisation number: TFA 2021-0146-2

Authorisation duration: From date signed by Minister's delegate, below, until 31 December 2022.

AUTHORISATION HOLDER

Joshua Roy Keen

Biota

Level 1, 288 Carr Place

Leederville WA 6007

AREA TO WHICH THIS AUTHORISATION APPLIES

Western Ridge, approximately 20 km SW of Newman, Millstream Chichester National Park and Ophthalmia Dam (Pilbara Region)

AUTHORISED ACTIVITY

Purpose of taking/disturbance:

To collect information needed to develop a robust monitoring program for Pilbara olive pythons at Western Ridge and to collect essential baseline ecological data from a known population (Millstream).

Threatened species authorised to be taken/disturbed (including conservation status):

Pilbara olive python, *Liasis olivaceus barroni* (Vulnerable)

Quantity of threatened species authorised to be taken/disturbed:

Up to 50 individual animals of the above listed threatened fauna species may potentially be captured and released during the trapping program.

Up to 30 individuals may have a VHF transmitter either surgically implanted or attached to their dorsal surface.

Authorised taking/disturbance methodology:

Take Pilbara olive pythons by hand or with snake hooks and bags. Captured pythons may have morphometric and condition details recorded (weight, sex, snout-vent length) and may be microchipped. Scale clips may be taken to provide a second means of identification and a DNA sample. Individuals may be palpated for no more than 10 minutes to encourage the provision of a faecal sample.

Suitable individuals may have a VHF transmitter weighing <3.6% of the individual's body weight surgically implanted using the methods detailed in Peter Spencer's Murdoch University Animal Ethics Committee approved project (RW3360/21). Transmitters will be surgically removed prior to battery

failure and may be replaced with a new transmitter. Pythons may be held for up to 24 hours (for the implantation or removal of transmitters and subsequent recovery) in clean black cloth bags inside an airconditioned office, with water made available *ad libatum*, before being released at the point of capture. The maximum time individuals may be fitted with transmitters is 3 years.

Very small pythons may have a VHF transmitter weighing <5% of the individual's body weight attached to the dorsal surface with cyanoacrylate glue.

At a minimum, pythons with implanted transmitters will be radio-tracked on bi-annual, two-week fieldtrips. Pythons with external transmitters may be radio-tracked multiple times within a 28 day period after release. Attempts to recapture individuals to remove or replace transmitters will occur when no less than 50-75% of the transmitter battery life is remaining.

All microchipping and scale-clipping will be completed by or under the direct supervision of the authorisation holder or a suitably qualified and experienced additional authorised person, as designated with an asterisk below. All radio-transmitter implantation and removal will be conducted by a suitably qualified, trained and experienced veterinarian, as designated with an asterisk below.

All proposed activities will be conducted in accordance with Animal Ethics Committee approval and DBCA Standard Operating Procedures (SOPs) for fauna survey and monitoring techniques.

ADDITIONAL AUTHORISED PERSONS

Roy Teale	Tim Oldfield*
Zoë Hamilton	Michael Greenham
Nathan Beerkens	Jason Alexander
Peter Spencer	

Additional personnel named on Peter Spencer's Murdoch University Animal Ethics Committee approved project (RW3360/21) who are suitably qualified and experienced in the authorised activities working under the direction of the authorisation holder.

Field assistants assisting with the authorised activities working under the direct supervision of the authorisation holder or suitably qualified and experienced named additional authorised person.

CONDITIONS

1. The written authorisation of the person in possession or occupation of the land accessed and upon which threatened fauna is taken or disturbed must:
   a) state location details (including lot or location number, street/road, suburb and local government authority);
   b) state land owner or occupier name, and contact phone number;
   c) specify the time period that the authorisation is valid for;
   d) be signed and dated; and
   e) be attached to this Authorisation to take or disturb threatened species at all times.
2. This Authorisation to take or disturb threatened species, and any other written authorisation or lawful authority which authorises the take or disturbance of fauna on specified locations for the authorised activities must be carried at all times while conducting authorised activities and be produced on demand by a wildlife officer.
3. Additional authorised persons who are not suitably qualified and experienced in the authorised activities, and field assistants assisting with the authorised activities, must be working under direct supervision of experienced and competent named authorised persons.
4. Any inadvertently captured species of non-target threatened fauna or non-threatened fauna (threatened fauna as defined in *Biodiversity Conservation Act 2016* Section 19) is to be released

immediately at the point of capture. Details of such fauna must be included in the fauna taking/disturbance return as required under this authorisation.

5. The authorisation holder, unless specified in the authorised activities, must not:
   a) release any threatened fauna in any area where it does not naturally occur;
   b) transfer threatened fauna to any other person or authority (other than the Western Australian Museum) unless the fauna is injured or abandoned fauna (condition 6); or
   c) dispose of the remains of threatened fauna in any manner likely to confuse the natural or present-day distribution of the species.
6. All threatened fauna injuries, unexpected deaths, unplanned euthanasia, and abandoned young or eggs, must be reported by the authorisation holder to the DBCA Wildlife Protection Branch, Wildlife Licensing Section (wildlifelicensing@dbca.wa.gov.au) to notify of the incident and for advice on treatment or disposal. All deceased threatened fauna must be offered to the Western Australian Museum.
7. To prevent any unnecessary collecting in this State, all specimens and material taken and retained under this authorisation, that remain at the conclusion of the activities, must be offered to the Western Australian Museum for loan, for inclusion in its collection, or made available to other persons involved in relevant scientific studies if so required.
8. The authorisation holder must create, compile and maintain records and information as required in a DBCA approved "Return of Fauna Taken/Disturbed" of all fauna taking/disturbance activities as they occur.
9. A DBCA approved "Return of Fauna Taken/Disturbed" must be completed in full (including nil taking/disturbance details) and submitted to DBCA Wildlife Licensing Section (wildlifelicensing@dbca.wa.gov.au) prior to the end of the authorisation duration and, if the authorisation duration is greater than 12 months, prior to the end of each annual period of the authorisation (from the date signed by the Minister's delegate) (refer to "Additional Information" section below). Where a licence to take or disturb fauna is issued in conjunction with this Authorisation to take or disturb threatened species, a combined "Return of Fauna Taken/Disturbed" may be completed and submitted.
10. A written report detailing the undertaken authorised activities, outcome, unintended incidents, injuries and mortalities of threatened fauna, implemented monitoring, mitigation and management, and explaining the records and information as required in a DBCA approved "Return of Fauna Taken/Disturbed" must be submitted, in addition to a "Return of Fauna Taken/Disturbed" to DBCA Wildlife Licensing Section (wildlifelicensing@dbca.wa.gov.au).

ADDITIONAL INFORMATION

1. Before undertaking the Authorised Activity, permission must be obtained from: (a) the owner or occupier of private land; or (b) the Department or Authority controlling Crown land, on which the Threatened Fauna occur. This includes obtaining the written endorsement from Department of Biodiversity, Conservation and Attractions (DBCA) if the authorised activity is proposed for land managed by DBCA.
2. This Authorisation to take or disturb threatened species does not constitute lawful authority issued under regulations 4 and 8 of the *Conservation and Land Management Regulations 2002*. Contact the applicable Department District Officer for further information.
3. The approved DBCA "Return of Fauna Taken/Disturbed" template can be obtained from DBCA Wildlife Licensing Section (wildlifelicensing@dbca.wa.gov.au).
4. Any interaction involving nationally listed threatened fauna that may be harmful to the fauna and/or invasive may require approval from the Commonwealth Department of the Environment and Energy (http://www.environment.gov.au/biodiversity/threatened/permits). Interaction with such species is controlled by the Commonwealth *Environment Protection and Biodiversity*

Conservation Act 1999 and *Environment Protection and Biodiversity Conservation Regulations 2000*.

5. It is the responsibility of the authorisation holder to ensure that they comply with the requirements of all applicable legislation.
6. An Authorisation to take or disturb threatened species does not constitute an animal ethics approval or a licence to use animals for scientific purposes as required under the *Animal Welfare Act 2002, Animal Welfare (Scientific Purposes) Regulations 2003*. Enquiries relating to the Animal Welfare Act scientific purposes licence and animal ethics committee approvals are to be directed to the Western Australian Department of Primary Industries and Regional Development (https://www.agric.wa.gov.au/animalwelfare).

Margaret Byrne

Dr Margaret Byrne

Executive Director of Biodiversity and Conservation Science

AS DELEGATE OF THE MINISTER

DATE: ..14../....2...../2022

Appendix 3

Ethics Permit

A/Prof Peter Spencer
College of Science, Health, Engineering and Education
Murdoch University

Murdoch University

Research and Innovation

Animal Ethics
Research Ethics & Integrity Office

90 South Street, Murdoch
Western Australia 6150

T +61 8 9360 7366

murdoch.edu.au

CRICOS Provider Code 00125J
ABN 61 616 369 313

Tuesday, 30 November 2021

Dear Peter,

ANIMAL ETHICS

Protocol ID.	898
Permit No.	**RW3360/21**
Protocol Title	Western Ridge Pilbara Olive Python monitoring

Thank you for your reply to the letter dated 15th November 2021 regarding the AEC response to the above Permit Application. The committee's concerns have all been addressed and the permit now has **OUTRIGHT** approval. Work using animals may commence.

Special Condition/s of Approval for this Permit

- The AEC has requested that a video of the surgical procedure to implant the transmitter in the snakes is provided to the animal ethics office, when available, for monitoring purposes.

The approval of this project requires you to adhere to the conditions outlined in this letter and to comply with the Animal Welfare Act (2002) and the *Australian code for the care and use of animals for scientific purposes* (8th edition, 2013).

Investigators must maintain records of the care and use of animals and Chief Investigators must provide to the AEC an Annual Report which is due in January each year.

Location	Impact	Species Code	Animal Species	Number Requested	Number Approved
WA	6. Major Surgery with recovery	41	Pilbara Olive Python (*Liasis olivaceus barroni*)_ transmitter fitted	30	30
WA	3. Minor conscious intervention	41	Pilbara Olive Python (*Liasis olivaceus barroni*) – captured and released	20	20

The Research Ethics and Integrity Office wish you every success for your research.

Dr Moira Desport
Animal Ethics Adviser
On behalf of the Animal Ethics Committee

cc: Roy Teale, Dr Zoe Hamilton, Nathan Beerkens, Joshua Keen and Dr Tim Oldfield

Standard Conditions for Teaching and Research

Responsibilities of Chief Investigators:
Investigators and teachers have personal responsibility for all matters related to the welfare of the animals they use and must act in accordance with all requirements of the Australian code for the care and use of animals for scientific purposes (current edition). This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project.

In addition, the AEC requires Chief Investigators to:

1. Provide the Research Ethics & Integrity Office with a copy of any current licences and permits associated with the project e.g. from Department of Biodiversity, Conservation and Attractions (DBCA); Fisheries; DPIRD etc.
2. Ensure all personnel associated with the project have completed Animal Care and Ethics (ACE) registration with the Research Ethics & Integrity Office.
3. Provide prompt notification to the Research Ethics & Integrity Office immediately any unforeseen or adverse event occurs.
4. Ensure accurate records of the use of animals are maintained.
5. Where personnel from other Institutions are involved in the project, or when premises of another Institution are being utilised, that Institution must be advised of the project and must provide approval or formally delegate approval of the proposal.

Permits:
- Permits are valid for three years from the date of AEC approval providing a satisfactory annual report is submitted and approved by February of each year.
- Permits may be closed by a Chief Investigator with the submission of a Closure Annual Report or by an AEC directive.
- Investigators may be added to a permit following the submission of an amendment form and providing the investigator meets ACE registration and competency requirements. All forms are available on the Research Ethics & Integrity website.
- Please quote your ethics permit number in all correspondence.

Permits are treated in confidence. To enable the institution to fulfil requirements under the Animal Welfare Act WA (2002), information contained in your permit may be released to appropriate personnel at any collaborating institution. In addition, selected information from the application may also be provided to authorised personnel within the appropriate School or Faculty at Murdoch University. Commercial or patentable information should be clearly separated and marked "Commercial-in-Confidence".

Licences:
The Licence to use animals for scientific purposes in WA is obtained from the Department of Prim and Regional Development (DPIRD) by the Research Ethics & Integrity Office on behalf of the U University is also licensed in most other states in Australia. It is a requirement that licences ar public scrutiny. Therefore, you must ensure that the relevant licence is:

a. Displayed wherever animals are used for scientific purposes, e.g. in your laboratory

b. Carried by investigators in the field, e.g. in the car or boat.

Adverse Events/Unexpected deaths:

All adverse events or unexpected deaths should be promptly reported to the Animal Welfare Officer on 0447 061 593. In the event of the death of an animal (including any that have a tracking device attached), the cadaver must be cooled immediately and refrigerated as soon as possible. Do not freeze the cadaver. Disable but do not remove any tracking devices. Unless exemption is specifically provided, cadavers must be independently examined. Murdoch Pathology is currently unable to conduct post-mortems. DPIRD is available to perform a PM as required by the *Australian code of practice for the care and use of animals for scientific purposes (2013)* and can be contacted on 0448 365 346. Additional costs may be involved. Please ensure you provide a reasonable amount of history of the circumstances of the animal death and request the return of any transmitters. The cadaver is to be transported with ice packs. The post-mortem report should be forwarded promptly to animal.ethics@murdoch.edu.au

Appendix 4

Field Effort Summary

1.0 Field Effort

1.1 Western Ridge

1.1.1 Phase 1

A total of two nights spotlighting with four people (Joshua Keen, Nathan Beerkens, Tim Oldfield and Dr. Zoë Hamilton) and a single night with five people (BHP employee Emma Stock included) yielded a total of 19 hours of effort (Table 1.1). Sites at Western Ridge were very dry with few rock pools.

Table 1.1: Location and effort of nocturnal Pilbara Olive Python searches within the Western Ridge survey area during phase one.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Nankunya (Afghan Spring)	-23.3831	119.6147	15/01/2022	90	4	360
Xanadu Gorges	-23.3898	119.6128	16/01/2022	120	4	480
Xanadu Gorges	-23.3898	119.6128	18/01/2022	30	5	150
Nankunya (Afghan Spring)	-23.3831	119.6147	18/01/2022	30	5	150
					Total	**1,140**

1.1.2 Phase 2

During the second phase of the survey POP sites were surveyed by spotlighting in the evening and into the later period of the night. A total of five nights were spent spotlighting at Western Ridge, four nights with three Biota personnel (Joshua Keen, Nathan Beerkens & Dr. Zoë Hamilton, 22/02/2022 – 25/02/2022) and a single night with four Biota personnel (Joshua Keen, Nathan Beerkens, Dr. Zoë Hamilton & Dr. Samuel Timothy Oldfield on 01/03/2022). This yielded a total of 41.5 hours of effort (Table 1.2).

Table 1.2. Location and effort of nocturnal Pilbara Olive Python searches within the Western Ridge area during phase two.

Location	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Xanadu Gorges	-23.3898	119.6128	22/02/2022	80	3	240
Nankunya (Afghan Spring)	-23.3831	119.6147	22/02/2022	45	3	135
Nankunya (Afghan Spring)	-23.3831	119.6147	23/02/2022	60	3	180
Xanadu Gorges	-23.3898	119.6128	23/02/2022	120	3	360
Nankunya (Afghan Spring)	-23.3831	119.6147	24/02/2022	60	3	180
Xanadu Gorges	-23.3898	119.6128	24/02/2022	120	3	360
Nankunya (Afghan Spring)	-23.3831	119.6147	25/02/2022	80	3	240
Xanadu Gorges	-23.3898	119.6128	25/02/2022	100	3	300
Nankunya (Afghan Spring)	-23.3831	119.6147	01/03/2022	45	4	180
Xanadu Gorges	-23.3898	119.6128	01/03/2022	80	4	320
					Total	**2,495**

1.1.3 Phase 3

Pop sites were surveyed by spotlighting in the evening and into the later period of the night.
A total of three nights were spent spotlighting at Western Ridge, two nights with two Biota personnel (Nathan Beerkens & Dr. Zoë Hamilton, 07/12/2022 – 08/12/2022) and a single night with three Biota personnel (Nathan Beerkens, Dr. Zoë Hamilton & Dr. Samuel Timothy Oldfield on 09/12/2022). This yielded a total of 889 mins of effort (see Table 1.3). Water was present in three main rock pools at Nankunya (Afghan Spring), but all previously sampled rock pools in Skeleton Gorge were dry.

Table 1.3. Location and effort of nocturnal Pilbara Olive Python searches within the Western Ridge survey area during phase three.

Location	Description	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Nankunya (Afghan Spring)	PoP searches	-23.3831	119.6147	07/12/2022	100	2	200
Nankunya (Afghan Spring)	PoP searches	-23.3831	119.6147	08/12/2022	82	2	164
Nankunya (Afghan Spring) & Xanadu/Skeleton gorges	PoP releases at Nankunya and radio-tracking of POP 201 across Xanadu and Skeleton gorges. PoP searches throughout.	-23.3831	119.6147	09/12/2022	175	3	525
						Total	**889**

1.1.4 Phase 4

POP sites were surveyed by spotlighting in the evening and into the later period of the night at the Western Ridge study area. A total of three nights were spent spotlighting at Western Ridge, a single night with four Biota personnel (Dr. Zoë Hamilton, Nathan Beerkens, Joshua Keen and Samuel Timothy Oldfield on 24/01/2023) and two nights with three Biota personnel (26/01/2023 and 27/01/2023). This yielded a total of 1,085 mins of effort (see Table 1.4). Water was present in two main rock pools at Nankunya (Afghan Spring), and one rock pool at Skeleton Gorge.

Table 1.4: Location and effort of nocturnal Pilbara Olive Python searches within the Western Ridge survey area during phase four.

Location	Description	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Western Ridge Nankunya (Afghan Spring) Skeleton Gorge	POP searches, eDNA sampling and radio tracking attempts at Nankunya. Radio-tracking of POP 201 along Skeleton gorge. Attempted to radio track POP 211and POP 212 but no signal detected.	-	-	24/01/2023	125	4	500
Western Ridge Nankunya (Afghan Spring) Xanadu Gorge East Skeleton Gorge West Skeleton Gorge	POP searches throughout, eDNA sampling and radio tracking radio-tracking of POP 201 to Skeleton gorge. Attempted to radio track POP 211and POP 212 but no signal detected.	-	-	26/01/2023	3	495	165
Western Ridge Nankunya (Afghan Spring) Xanadu Gorge Skeleton Gorge	POP searches at Nankunya and Xanadu, POP release at Nankunya and radio tracking radio-tracking of POP 201 back to Skeleton gorge. Attempted to radio track POP 211and POP 212 but no signal detected.	-	-	27/01/2023	140	3	420
						Total	**1,085**

1.1.5 Phase 5

POP sites were surveyed by traversing and spotlighting in the evening and into the later period of the night at the Western Ridge study area. A total of six nights were spent spotlighting at Western Ridge, with a team of two zoologists. This yielded a total of 1,784 mins of effort (see Table 1.5). Water was present in two main rock pools at Nankunya (Afghan Spring), and one rock pool at Skeleton Gorge.

Table 1.5. Location and effort of nocturnal Pilbara Olive Python searches within the Western Ridge survey area during phase five.

Location	Description	Date	Duration (mins)	No. of Observers	Effort (mins)
Western Ridge Nankunya (Afghan Spring) Xanadu Gorge	POP searches, and radio tracking attempts at Nankunya. Radio-tracking of POP POP **216** to underneath rock at entrance of gorge. Attempts to detect POP 201, 211 and 212 but not detected.	9/05/2023	126	2	252
Western Ridge Nankunya (Afghan Spring) Xanadu Gorge East Skeleton Gorge West Skeleton Gorge Zion (new) Gorge	POP searches throughout, eDNA sampling and radio tracking radio-tracking of POP **216** to underneath same rock. Attempts to detect POP 201, 211 and 212 but not detected.	10/05/2023	180	2	360
Western Ridge Nankunya (Afghan Spring) Xanadu Gorge Skeleton Gorge	POP searches and radio-tracking at Nankunya and Xanadu, Skeleton Gorges. Radio-tracking and locating POP **216** in open in ambush position. Attempts to detect POP 201, 211 and 212 but not detected.	11/05/2023	266	2	532
Western Ridge Nankunya (Afghan	POP searches and radiotracking throughout. Radio-tracking and location of POP **216** at Nankunya, to higher in the escarpment. Deployed temperature loggers. POP **211** detected, tracked and located at North facing rock wall above Nankunya. Attempts to detect POP 201 and 212 but not detected.	12/05/2023	145	2	290
Western Ridge Nankunya (Afghan	Attempted to detect POP 211 again, no signal detected. Radio-tracked and located POP **216** further up the rocky escarpment. Checked last known location of POP 211 and not present Number of attempts for remaining POP (POP 201, 211 and 212) at various locations around WR.	13/05/2023	95	2	190
Western Ridge Nankunya (Afghan	Retrieved temperature loggers.	14/05/2023	80	2	160
				Total	**1,784**

1.1.6 Summary

In total of 128.7 hours (7,723 minutes) of effort was expended at Western Ridge over the five phases of field work, yielding seven POP records during the survey (phases 1 to 5 combined) (Figure 1.1).

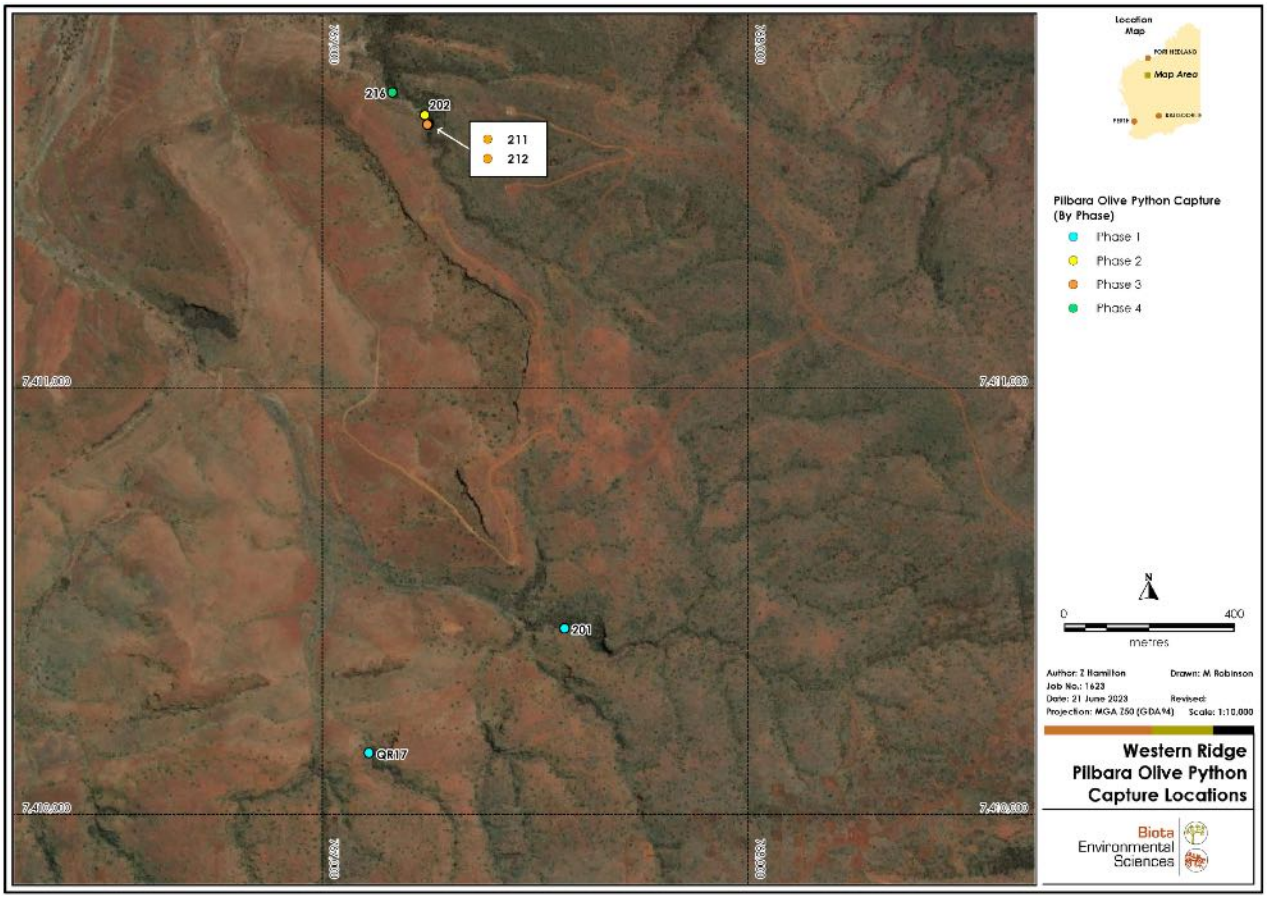

Figure 1.1. Western Ridge POP Records – all phases.

1.2 Millstream

1.2.1 Phase 1

Sites considered suitable for olive pythons were ground-truthed and searched for pythons early in the morning and then extensively searched using spotlighting from dusk until late into the night. Three nights of spotlighting at Millstream with a team of five personnel (Biota team + Hamish (DBCA)) for one night, and six personnel for two nights (Biota team + Jared & Matt - BHP) yielded a total of six Pilbara Olive Pythons, of which three were male and three were female Figure 1.2). The two additional BHPIO personnel contributed to a total of 31.5 hours of search effort at Millstream (see Table 1.6).

Table 1.6: Location and effort of nocturnal Pilbara Olive Python searches within the Millstream survey area during phase one.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Millstream Creek	-21.5895	117.0708	11/01/2022	60	6	360
Palm Pool Crossing	-21.5687	117.0524	11/01/2022	60	6	360
Millstream Creek	-21.5895	117.0708	12/01/2022	30	6	180
Palm Pool Crossing	-21.5687	117.0524	12/01/2022	90	6	540
Millstream Creek	-21.5895	117.0708	13/01/2022	30	5	150
Deep Reach Pool – picnic area	-21.6071	117.1060	13/01/2022	30	5	150
Deep Reach Pool – old campground	-21.6157	117.1102	13/01/2022	30	5	150
					Total	**1,890**

1.2.2 Phase 6

During this survey phase, the Millstream area was surveyed by radio-tracking each day as well as spot-lighting and radiotracking each evening for the duration of the survey (22nd to 26th May). Four days and evenings of targeted searches with a team of three zoologists yielded a total of 2,163 minutes of effort (see Table 1.7). No new, previously undetected Pilbara Olive Pythons were detected during this survey.

Table 1.7: Location and effort of nocturnal Pilbara Olive Python searches within the Millstream survey area during phase six.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Millstream homestead and along creek edge up to Central Hill.	-21.5895	117.0708	22/05/2023	102	3	306
Millstream – Palm Crossing	-21.5687	117.0524	23/05/2023	254	3	762
Millstream entirety	-21.5895	117.0708	24/05/2023	240	3	720
Millstream entirety	-21.5687	117.0524	25/05/2023	105	3	315
Python Pool	-21.5895	117.0708	26/05/2023	20	3	60
					Total	**2,163**

1.2.3 Summary

In total of 67.5 hours (4,053 minutes) of effort was expended at Millstream over the two phases of monitoring, yielding six individual POP records (Figure 1.2).

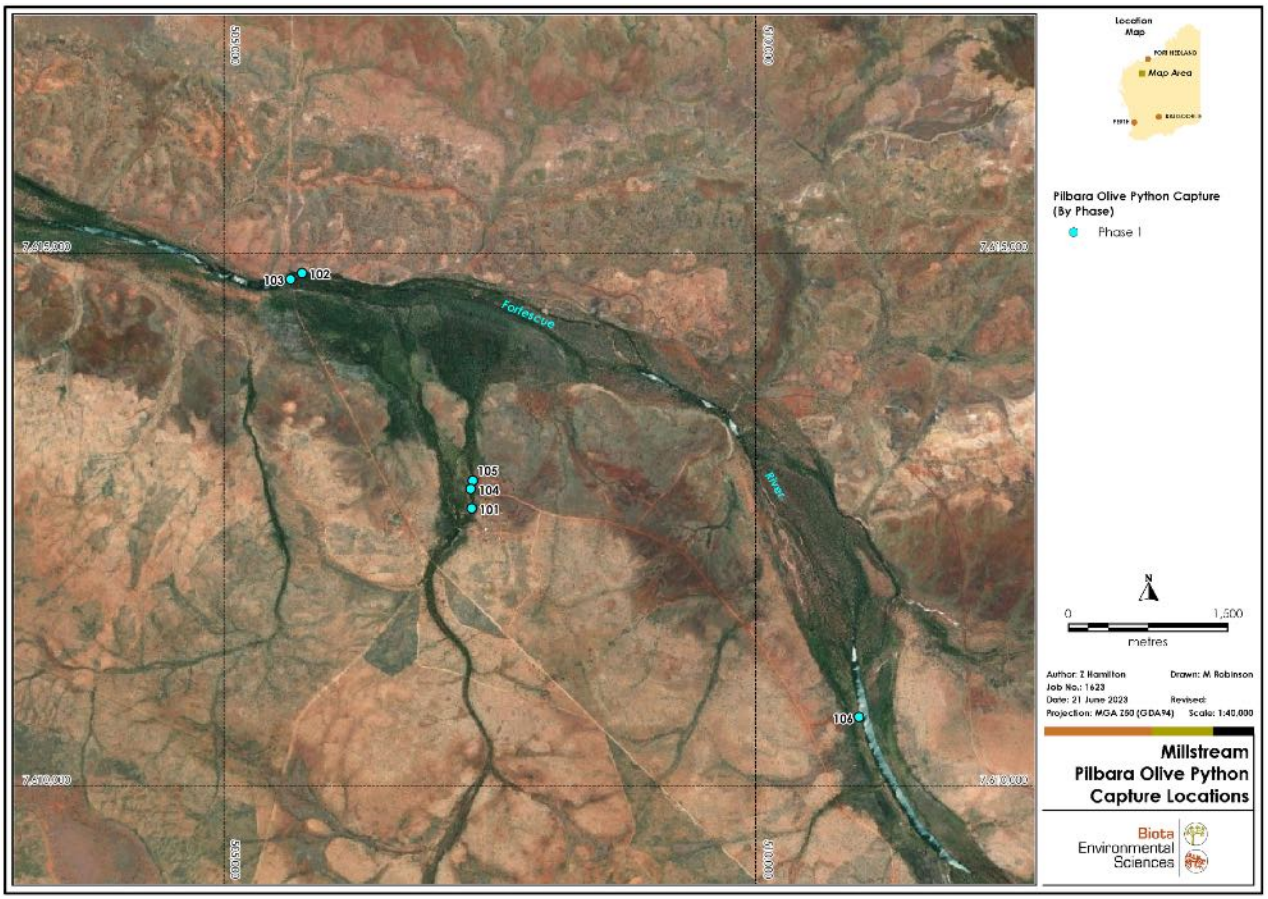

Figure 1.2: Location of Pilbara Olive Pythons recorded at Millstream-Chichester National Park

All live capture records were obtained during phase 1.

1.3 Ophthalmia Dam

1.3.1 Phase 2

POP sites at Ophthalmia were again surveyed by spotlighting in the evening and into the later period of the night. A total of three nights of spotlighting with four Biota personnel (Joshua Keen, Nathan Beerkens, Dr. Zoë Hamilton & Samuel Timothy Oldfield, 26/02/2022 to 28/02/2022), in addition to one short night focussed on releases and radio-tracking of tagged previously-tagged individuals (02/03/2022) yielded a total of 44 hours of effort (see Table 1.8). Eight individual POP were captured during the 44 hours expended at Ophthalmia Dam (Figure 1.3). All individuals were measured, scale-marked, tissue retained for genetic analysis and fitted with radio-telemetry transmitters during Phase two.

Table 1.8: Location and effort of nocturnal Pilbara Olive Python searches within the Ophthalmia Dam survey area during phase one.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Wall A	-23.3411	119.8592	26/2/2022	120	4	480
Wall C	-23.3388	119.8799	26/2/2022	45	4	180
Creek crossing	-23.3147	119.8706	26/2/2022	15	4	60
Eastern swamp	-23.3521	119.8995	26/2/2022	45	4	180
Eastern swamp	-23.3521	119.8995	27/2/2022	65	4	260
Wall C	-23.3388	119.8799	27/2/2022	45	4	180
Public area	-23.3397	119.8774	27/2/2022	25	4	100
Creek crossing	-23.3147	119.8706	27/2/2022	60	4	240
Wall B	-23.3387	119.8752	28/2/2022	60	4	240
Eastern swamp	-23.3521	119.8995	28/2/2022	60	4	240
Wall C	-23.3388	119.8799	28/2/2022	45	4	180
Creek crossing	-23.3147	119.8706	28/2/2022	45	4	180
Eastern swamp	-23.3521	119.8995	2/3/2022	10	4	40
Creek crossing	-23.3147	119.8706	2/3/2022	40	2	80
					Total	**2,640**

1.3.2 Phase 3

POP sites at Ophthalmia were also surveyed by spotlighting in the evening and into the later period of the night. A single night of spotlighting with two Biota personnel (Nathan Beerkens & Dr. Zoë Hamilton 10/12/2022) yielded a total of 410 minutes of effort (see Table 1.9). Two new Pilbara Olive Pythons were captured from Ophthalmia Dam. These two pythons were measured, and scale clips taken before releasing back at point of capture. No radio transmitters were implanted into these pythons during this phase.

Table 1.9: Location and effort of nocturnal Pilbara Olive Python searches within the Ophthalmia Dam survey area during phase three.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Ophthalmia Dam Swamp	-23.3521	119.8995	10/12/2022	160	2	320
Ophthalmia Dam	-23.3147	119.8706	10/12/2022	45	2	90
					Total	**410**

1.3.3 Phase 4

POP sites at the Ophthalmia Dam survey area were also surveyed by spotlighting in the evening and into the later period of the night. Four nights of spotlighting with four Biota personnel (Dr. Zoë Hamilton, Nathan Beerkens, Joshua Keen and Timothy Oldfield) yielded a total of 1,492 minutes of effort (see Table 1.10). Two new previously undetected Pilbara Olive Pythons, and one recapture from the December survey were captured from Ophthalmia Dam. These three pythons had radio trackers implanted, were measured and scale clips taken before releasing back at point of capture.

Table 1.10: Location and effort of nocturnal Pilbara Olive Python searches within Ophthalmia Dam survey area during phase four.

Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Eastern Swamp	-23.3521	119.8995	23/01/2023	109	4	436
Eastern Swamp	-23.3521	119.8995	25/01/2023	104	4	416
Eastern Swamp	-23.3521	119.8995	28/01/2023	115	4	460
Eastern Swamp	-23.3521	119.8995	29/01/2023	45	4	180
					Total	**1,492**

1.3.4 Phase 5

The Ophthalmia Dam study area was surveyed by spotlighting and radio-tracking each evening of the survey. The six nights of targeted searches each with a team of two zoologists yielded a total of 1,716 minutes of effort (28.6 hours, see Table 1.11). Two previously undetected POP were recorded, one live individual and one which was a carcass in the later stages of decomposition. In addition, a suspected POP slough was collected. The one new live python was implanted with a radio tracker, measured and scale clips taken before releasing back at the point of capture. Six pythons (POP **203**, **204, 206**, **207**, **214**, **215**) were tracked and recaptured. One of these individuals (POP **203**) had the radio transmitter surgically removed and then replaced, as battery life was reaching its end for transmission.

Table 1.11: Location and effort of nocturnal Pilbara Olive Python searches within the Ophthalmia Dam survey area during phase five

.Site	Latitude	Longitude	Date	Duration (mins)	No. of Observers	Effort (mins)
Ophthalmia Dam Typha swamp	-23.3521	119.8995	09/05/2023	140	2	280
Ophthalmia Dam Typha swamp	-23.3521	119.8995	10/05/2023	207	2	414
Ophthalmia Dam Typha Swamp Pools	-23.3521	119.8995	11/05/2023	141	2	282
Ophthalmia Dam Typha Swamp Pools	-23.3521	119.8995	12/05/2023	180	2	360
Eastern Swamp	-23.3521	119.8995	13/05/2023	120	2	240
Ophthalmia Dam Typha swamp	-23.3521	119.8995	13/05/2023	120	2	240
					Total	**1,716**

1.3.5 Summary

In total of 104.3 hours (6,258 minutes) of effort was expended at Ophthalmia Dam over the four phases of field work yielding fourteen POP records during the survey (phases 2, 3, 4 and 5 combined) (Figure 1.3).

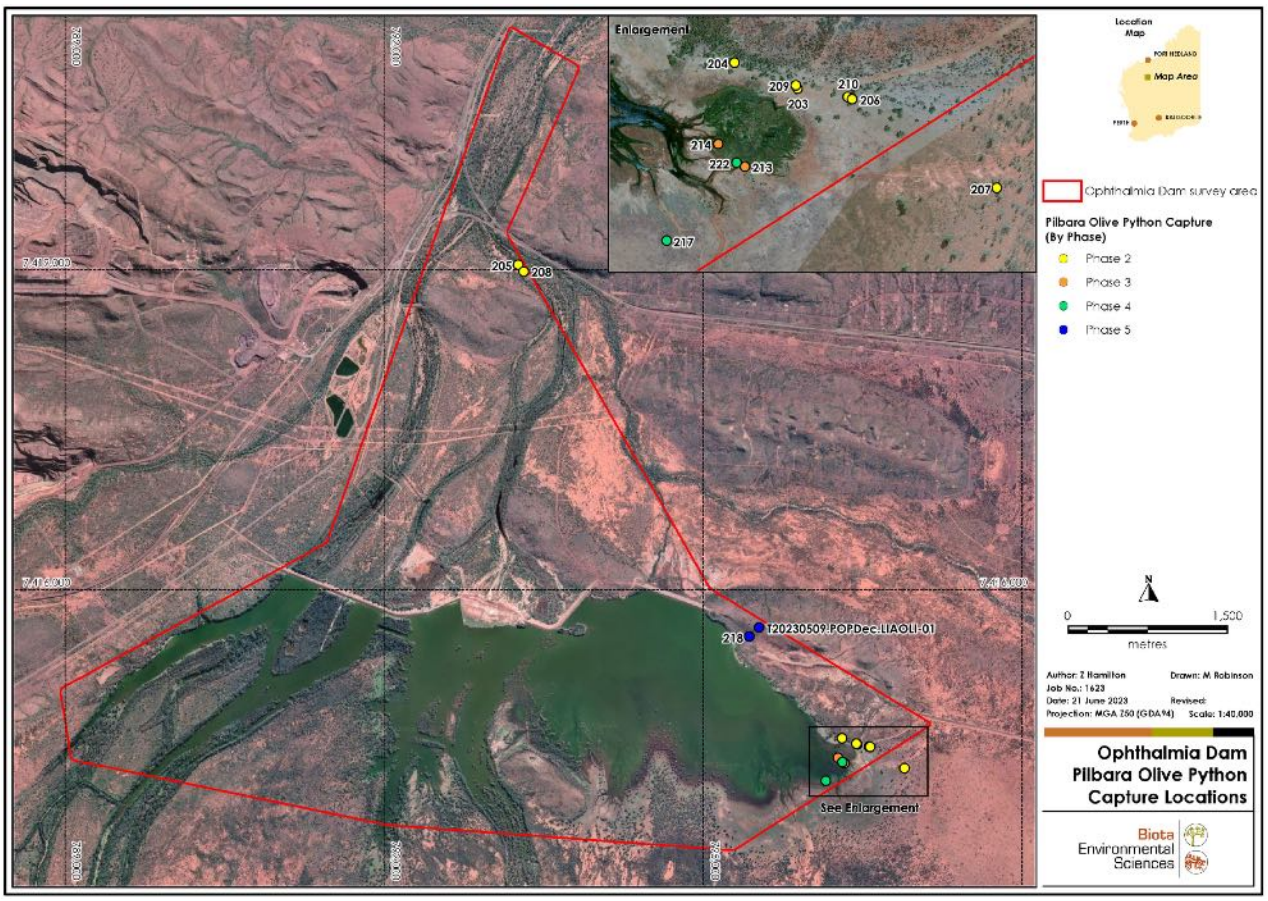

Figure 1.3: Location of Pilbara Olive Pythons recorded at Ophthalmia Dam.

Appendix 5

Helix eDNA Sampling Results

Table 1. Summary of eDNA samples and results from Phase 1; Millstream.

Location	Replicate	Site Code	Latitude	Longitude	Filtered Date	Phase	16S metabarcoding result
Palm Crossing	1	POP01-TS_ZH	-21.5703995	117.0546437	11/1/2022	1	Negative
Palm Crossing	2	POP01-TS_ZH	-21.5703995	117.0546437	11/1/2022	1	Negative
Palm Crossing	3	POP01-TS_ZH	-21.5703995	117.0546437	11/1/2022	1	Negative
Millstream 1	1	POP01TS_NB	-21.5740477	117.0822022	11/1/2022	1	Negative
Millstream 1	2	POP01TS_NB	-21.5740477	117.0822022	11/1/2022	1	Negative
Millstream 1	3	POP01TS_NB	-21.5740477	117.0822022	11/1/2022	1	Negative
Millstream 2	1	POP02-TS_ZH	-21.5774468	117.0815432	11/1/2022	1	Negative
Millstream 2	2	POP02-TS_ZH	-21.5774468	117.0815432	11/1/2022	1	Negative
Millstream 2	3	POP02-TS_ZH	-21.5774468	117.0815432	11/1/2022	1	Negative
Millstream	1	POP01_OPP	Locality information lost	Locality information lost	12/1/2022	1	Negative
Millstream	2	POP01_OPP	Locality information lost	Locality information lost	12/1/2022	1	**Positive**
Millstream	3	POP01_OPP	Locality information lost	Locality information lost	12/1/2022	1	Negative
Palm Crossing 2	1	POP02-TS_JKE	-21.569452	117.055357	12/1/2022	1	Negative
Palm Crossing 2	2	POP02-TS_JKE	-21.569452	117.055357	12/1/2022	1	Negative
Palm Crossing 2	3	POP02-TS_JKE	-21.569452	117.055357	12/1/2022	1	Negative
Deep Reach	1	POP02TS_NB	-23.383323	119.614581	12/1/2022	1	Negative
Deep Reach	2	POP02TS_NB	-23.383323	119.614581	12/1/2022	1	Negative
Deep Reach	3	POP02TS_NB	-23.383323	119.614581	12/1/2022	1	Negative

Table 2. Summary of eDNA samples and results from Phase 1; Western Ridge.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result
Xanadu	1	POPWR01_TS_NB	-23.3945038	119.6191891	15/01/2022	1	Negative
Xanadu	3	POPWR01_TS_NB	-23.3945038	119.6191891	15/01/2022	1	Negative
Afghan Springs / Nankunya	1	POPWR02-TS_NB	-23.383323	119.614581	15/01/2022	1	Negative
Afghan Springs / Nankunya	2	POPWR02-TS_NB	-23.383323	119.614581	15/01/2022	1	Negative
Skeleton East - 1	1	**POPWR03_TS_NB**	-23.3967784	119.6135118	16/01/2022	1	**Positive**
Skeleton East - 1	2	POPWR03_TS_NB	-23.3967784	119.6135118	16/01/2022	1	Negative
Skeleton East - 1	3	POPWR03_TS_NB	-23.3967784	119.6135118	16/01/2022	1	Negative
Skeleton East -2	1	POPWR04_TS_JKE	-23.39717968	119.6140531	16/01/2022	1	Negative
Skeleton East -2	2	POPWR04_TS_JKE	-23.39717968	119.6140531	16/01/2022	1	Negative
Skeleton East -2	3	POPWR04_TS_JKE	-23.39717968	119.6140531	16/01/2022	1	Negative

Table 3. Summary of eDNA samples and results from Phase 2; Western Ridge.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result
Afghan Springs / Nankunya	1	POP02TS_NB	-23.383323	119.614581	24/2/2022	2	Negative

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result
Afghan Springs / Nankunya	2	**POP02TS_NB**	-23.383323	119.614581	24/2/2022	2	**Positive**
Afghan Springs / Nankunya	3	POP02TS_NB	-23.383323	119.614581	24/2/2022	2	Negative
Skeleton East - 1	1	**POP08TS_ZH**	-23.396756	119.613532	25/2/2022	2	**Positive**
Skeleton East - 1	2	**POP08TS_ZH**	-23.396756	119.613532	25/2/2022	2	**Positive**
Skeleton East - 1	3	POP08TS_ZH	-23.396756	119.613532	25/2/2022	2	Negative
Afghan Springs / Nankunya	1	POP03TS_NB	-23.383323	119.614581	25/2/2022	2	Negative
Afghan Springs / Nankunya	2	POP03TS_NB	-23.383323	119.614581	25/2/2022	2	Negative
Afghan Springs / Nankunya	3	POP03TS_NB	-23.383323	119.614581	25/2/2022	2	Negative
Afghan Springs / Nankunya	1	**POP09TSEDNA_ZH**	-23.383323	119.614581	26/2/2022	2	**Positive**
Afghan Springs / Nankunya	2	**POP09TSEDNA_ZH**	-23.383323	119.614581	26/2/2022	2	**Positive**
Afghan Springs / Nankunya	3	**POP09TSEDNA_ZH**	-23.383323	119.614581	26/2/2022	2	**Positive**
Skeleton West	1	POP21TSEDNA_JKE	-23.3970038267	119.612113657	26/2/2022	2	Negative
Skeleton West	2	POP21TSEDNA_JKE	-23.3970038267	119.612113657	26/2/2022	2	Negative
Skeleton West	3	POP21TSEDNA_JKE	-23.3970038267	119.612113657	26/2/2022	2	Negative
Skeleton East - 1	1	**POP22TS_JKE**	-23.396756	119.613532	26/2/2022	2	**Positive**
Skeleton East - 1	2	**POP22TS_JKE**	-23.396756	119.613532	26/2/2022	2	**Positive**
Skeleton East - 1	3	**POP22TS_JKE**	-23.396756	119.613532	26/2/2022	2	**Positive**

Table 4. Summary of eDNA samples and results from Phase 4; Western Ridge and Ophthalmia Dam.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result
Ophthalmia Dam	1	**EDNA_20230125_OD07**	-23.3332435977	119.865583113	25/01/2023	4	**Positive**
Ophthalmia Dam	2	EDNA_20230125_OD07	-23.3332435977	119.865583113	25/01/2023	4	Negative
Ophthalmia Dam	3	EDNA_20230125_OD07	-23.3332435977	119.865583113	25/01/2023	4	Negative
Ophthalmia Dam	1	eDNA_202230125_OD06	-23.3337870958	119.858782441	25/01/2023	4	Negative
Ophthalmia Dam	2	eDNA_202230125_OD06	-23.3337870958	119.858782441	25/01/2023	4	Negative
Ophthalmia Dam	3	eDNA_202230125_OD06	-23.3337870958	119.858782441	25/01/2023	4	Negative
Ophthalmia Dam	1	**eDNA_20230125_OD05**	-23.3327594276	119.854618635	25/01/2023	4	**Positive**
Ophthalmia Dam	2	eDNA_20230125_OD05	-23.3327594276	119.854618635	25/01/2023	4	Negative
Ophthalmia Dam	3	eDNA_20230125_OD05	-23.3327594276	119.854618635	25/01/2023	4	Negative
Ophthalmia Dam	1	eDNA20230125.OD3	-23.3530834	119.8977907	25/01/2023	4	Negative
Ophthalmia Dam	2	eDNA20230125.OD3	-23.3530834	119.8977907	25/01/2023	4	Negative
Ophthalmia Dam	3	eDNA20230125.OD3	-23.3530834	119.8977907	25/01/2023	4	Negative
Ophthalmia Dam	1	eDNA20230125.OD2	-23.3522114	119.8989417	25/01/2023	4	Negative
Ophthalmia Dam	2	eDNA20230125.OD2	-23.3522114	119.8989417	25/01/2023	4	Negative
Ophthalmia Dam	3	eDNA20230125.OD2	-23.3522114	119.8989417	25/01/2023	4	Negative
Ophthalmia Dam	1	eDNA20230125.OD1	-23.3524013	119.8973509	25/01/2023	4	Negative
Ophthalmia Dam	2	eDNA20230125.OD1	-23.3524013	119.8973509	25/01/2023	4	Negative
Ophthalmia Dam	3	eDNA20230125.OD1	-23.3524013	119.8973509	25/01/2023	4	Negative

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result
Western Ridge, Skeleton Gorge	1	1623_20230124_SKEL01	-23.39719334	119.61403789	25/01/2023	4	Negative
Western Ridge, Skeleton Gorge	2	1623_20230124_SKEL01	-23.39719334	119.61403789	25/01/2023	4	Negative
Western Ridge, Skeleton Gorge	3	1623_20230124_SKEL01	-23.39719334	119.61403789	25/01/2023	4	Negative
Western Ridge, Nankunya	1	eDNA20230124.NAN02	-23.3824197	119.6134781	24/01/2023	4	Negative
Western Ridge, Nankunya	2	eDNA20230124.NAN02	-23.3824197	119.6134781	24/01/2023	4	Negative
Western Ridge, Nankunya	3	eDNA20230124.NAN02	-23.3824197	119.6134781	24/01/2023	4	Negative
Western Ridge, Nankunya	1	eDNA20230126.NAN02	-23.3590762597	119.734765627	26/01/2023	4	Negative
Western Ridge, Nankunya	2	eDNA20230126.NAN02	-23.3590762597	119.734765627	26/01/2023	4	Negative
Western Ridge, Nankunya	3	**eDNA20230126.NAN02**	-23.3590762597	119.734765627	26/01/2023	4	**Positive**
Western Ridge, Nankunya	4	**eDNA20230126.NAN02**	-23.3590762597	119.734765627	26/01/2023	4	**Positive**
Western Ridge, Nankunya	5	**eDNA20230126.NAN02**	-23.3590762597	119.734765627	26/01/2023	4	**Positive**
Western Ridge, Nankunya	6	eDNA20230126.NAN02	-23.3590762597	119.734765627	26/01/2023	4	Negative

Table 5. Summary of eDNA samples and results from Phase 5; Western Ridge and Ophthalmia Dam.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result	Helix eDNA probe result
Ophthalmia Dam	1	1623_20230512_OD01	-23.3518268	119.9005493	12/5/2023	5	Negative	Negative
Ophthalmia Dam	2	1623_20230512_OD01	-23.3518268	119.9005493	12/5/2023	5	Negative	**Positive**
Ophthalmia Dam	3	1623_20230512_OD01	-23.3518268	119.9005493	12/5/2023	5	Negative	**Positive**
Ophthalmia Dam	1	1623_20230513_OD02	-23.3518268	119.865983	13/5/2023	5	Negative	Negative
Ophthalmia Dam	2	1623_20230513_OD02	-23.3518268	119.865983	13/5/2023	5	Negative	Negative
Ophthalmia Dam	3	1623_20230513_OD02	-23.3518268	119.865983	13/5/2023	5	Negative	Negative
Ophthalmia Dam	1	**1623_20230512_OD5**	-23.3323339	119.8542285	12/5/2023	5	**Positive**	**Positive**
Ophthalmia Dam	2	1623_20230512_OD5	-23.3323339	119.8542285	12/5/2023	5	Negative	**Positive**
Ophthalmia Dam	3	**1623_20230512_OD5**	-23.3323339	119.8542285	12/5/2023	5	**Positive**	**Positive**
Ophthalmia Dam	1	**eDNA_202230125_OD06**	-23.3337870958	119.858782441	25/01/2023	5	**Positive**	Negative
Ophthalmia Dam	2	eDNA_202230125_OD06	-23.3337870958	119.858782441	25/01/2023	5	Negative	**Positive**
Ophthalmia Dam	3	eDNA_202230125_OD06	-23.3337870958	119.858782441	25/01/2023	5	Negative	Negative
Ophthalmia Dam	1	1623_20230510_ODNW	-23.3147797	119.870395	10/5/2023	5	Negative	Negative
Ophthalmia Dam	2	1623_20230510_ODNW	-23.3147797	119.870395	10/5/2023	5	Negative	Negative
Ophthalmia Dam	3	1623_20230510_ODNW	-23.3147797	119.870395	10/5/2023	5	Negative	Negative
Ophthalmia Dam	1	1623_20230510_ODTypha	23.3518487	119.9004386	10/5/2023	5	Negative	Negative
Ophthalmia Dam	2	**1623_20230510_ODTypha**	23.3518487	119.9004386	10/5/2023	5	**Positive**	Negative
Ophthalmia Dam	3	1623_20230510_ODTypha	23.3518487	119.9004386	10/5/2023	5	Negative	Negative
Ophthalmia Dam	1	1623_20230510_ODNE	-23.3145708648	119.872707948	10/5/2023	5	Negative	Negative
Ophthalmia Dam	2	1623_20230510_ODNE	-23.3145708648	119.872707948	10/5/2023	5	Negative	Negative
Ophthalmia Dam	3	**1623_20230510_ODNE**	-23.3145708648	119.872707948	10/5/2023	5	**Positive**	Negative

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result	Helix eDNA probe result
Western Ridge, Skeleton Gorge	1	Skeleton East / 1623_20230510_SE01	-23.3967993786	119.613525756	10/5/2023	5	Negative	Negative
Western Ridge, Skeleton Gorge	2	Skeleton East / 1623_20230510_SE01	-23.3967993786	119.613525756	10/5/2023	5	Negative	Negative
Western Ridge, Skeleton Gorge	3	Skeleton East / 1623_20230510_SE01	-23.3967993786	119.613525756	10/5/2023	5	Negative	Negative
Western Ridge	1	1623_20230510_WR01	-23.3961	119.6161	10/05/2023	5	Negative	Negative
Western Ridge	2	1623_20230510_WR01	-23.3961	119.6161	10/05/2023	5	Negative	Negative
Western Ridge	3	1623_20230510_WR01	-23.3961	119.6161	10/05/2023	5	Negative	Negative

Table 6. Summary of eDNA samples and results from Phase 6; Millstream.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	16S metabarcoding result	Helix eDNA probe result
Millstream	1	1623-20230525_millstream_zh05	-21.5704760562	117.061400397	25/5/2023	6	Negative	Negative
Millstream	2	1623-20230525_millstream_zh05	-21.5704760562	117.061400397	25/5/2023	6	Negative	**Positive**
Millstream	3	1623-20230525_millstream_zh05	-21.5704760562	117.061400397	25/5/2023	6	Negative	**Positive**
Millstream	1	1623_20230525_millstream_zh06	-21.5697505259	117.052640334	25/5/2023	6	Negative	Negative
Millstream	2	1623_20230525_millstream_zh06	-21.5697505259	117.052640334	25/5/2023	6	Negative	Negative
Millstream	3	1623_20230525_millstream_zh06	-21.5697505259	117.052640334	25/5/2023	6	Negative	Negative
Millstream	1	MillstreamZH03	-21.5705354	117.0546792	25/5/2023	6	Negative	**Positive**
Millstream	2	MillstreamZH03	-21.5705354	117.0546792	25/5/2023	6	Negative	**Positive**
Millstream	3	MillstreamZH03	-21.5705354	117.0546792	25/5/2023	6	Negative	**Positive**
Millstream	1	1623-20230523-millstream-zh01	-21.5920347671	117.069174967	25/5/2023	6	Negative	Negative
Millstream	2	1623-20230523-millstream-zh01	-21.5920347671	117.069174967	25/5/2023	6	Negative	**Positive**
Millstream	3	1623-20230523-millstream-zh01	-21.5920347671	117.069174967	25/5/2023	6	Negative	Negative
Millstream	1	MillstreamZH02	-21.5812781	117.0940686	25/5/2023	6	Negative	Negative
Millstream	2	MillstreamZH02	-21.5812781	117.0940686	25/5/2023	6	Negative	Negative
Millstream	3	MillstreamZH02	-21.5812781	117.0940686	25/5/2023	6	Negative	Negative

Table 7. Temporal eDNA sampling at Nankunya (Afghan Springs) Pool; Phase 2.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	eDNA result for POP
Afghan Springs / Nankunya	1	POP02TS_NB_01	-23.3833234	119.614581	24/2/2022	2	Negative
Afghan Springs / Nankunya	2	**POP02TS_NB_02**	-23.3833234	119.614581	24/2/2022	2	**Positive**
Afghan Springs / Nankunya	3	POP02TS_NB_03	-23.3833234	119.614581	24/2/2022	2	Negative
Afghan Springs / Nankunya	1	(POP03TS_NB) 01	-23.3387652	119.879914	25/2/2022	2	Negative
Afghan Springs / Nankunya	2	(POP03TS_NB) 02	-23.3387652	119.879914	25/2/2022	2	Negative
Afghan Springs / Nankunya	3	(POP03TS_NB) 03	-23.3387652	119.879914	25/2/2022	2	Negative
Afghan Springs / Nankunya	1	**POP09TSEDNA_ZH_01**	-23.3831952	119.61456	26/2/2022	2	**Positive**
Afghan Springs / Nankunya	2	**POP09TSEDNA_ZH_02**	-23.3831952	119.61456	26/2/2022	2	**Positive**
Afghan Springs / Nankunya	3	**POP09TSEDNA_ZH_03**	-23.3831952	119.61456	26/2/2022	2	**Positive**

Table 8. Temporal eDNA sampling at Nankunya (Afghan Springs) Pool; Phase 4.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	eDNA result for POP
Western Ridge, Nankunya	1	EDNA.20230127.nan01	-23.3832588662	119.614568726	2023/01/27	4	Negative
Western Ridge, Nankunya	2	EDNA.20230127.nan01	-23.3832588662	119.614568726	2023/01/27	4	Negative
Western Ridge, Nankunya	3	EDNA.20230127.nan01	-23.3832588662	119.614568726	2023/01/27	4	Negative
Western Ridge, Nankunya	1	EDNA_20230126_Nan01	-23.3833120298	119.61453544	2023/01/26	4	Negative
Western Ridge, Nankunya	2	EDNA_20230126_Nan01	-23.3833120298	119.61453544	2023/01/26	4	Negative
Western Ridge, Nankunya	3	**EDNA_20230126_Nan01**	-23.3833120298	119.61453544	2023/01/26	4	**Positive**
Western Ridge, Nankunya	4	**EDNA_20230126_Nan01**	-23.3833120298	119.61453544	2023/01/26	4	**Positive**
Western Ridge, Nankunya	5	**EDNA_20230126_Nan01**	-23.3833120298	119.61453544	2023/01/26	4	**Positive**
Western Ridge, Nankunya	5	EDNA_20230126_Nan01	-23.3833120298	119.61453544	2023/01/26	4	Negative
Western Ridge, Nankunya	1	eDNA20230124.NAN01	-23.3832813	119.6146086	2023/01/24	4	Negative
Western Ridge, Nankunya	2	eDNA20230124.NAN01	-23.3832813	119.6146086	2023/01/24	4	Negative
Western Ridge, Nankunya	3	eDNA20230124.NAN01	-23.3832813	119.6146086	2023/01/24	4	Negative

Table 9. Temporal eDNA sampling at Nankunya (Afghan Springs) Pool; Phase 5.

Location	Replicate	Site Code	Latitude	Longitude	Date	Phase	eDNA result for POP	Helix eDNA probe result
Western Ridge, Nankunya	1	1623_20230513_NAN01	-23.3833193	119.6145083	13/05/2023	5	Negative	Negative
Western Ridge, Nankunya	2	1623_20230513_NAN01	-23.3833193	119.6145083	13/05/2023	5	Negative	Negative
Western Ridge, Nankunya	3	1623_20230513_NAN01	-23.3833193	119.6145083	13/05/2023	5	Negative	**Positive**
Western Ridge, Nankunya	1	**1623_20230512_NAN01**	-23.3831990758	119.614590125	12/05/2023	5	**Positive**	**Positive**
Western Ridge, Nankunya	2	1623_20230512_NAN01	-23.3831990758	119.614590125	12/05/2023	5	Negative	**Positive**
Western Ridge, Nankunya	3	1623_20230512_NAN01	-23.3831990758	119.614590125	12/05/2023	5	Negative	**Positive**
Western Ridge, Nankunya	1	1623_20230510_NAN01	-23.3831990758	119.614590125	10/05/2023	5	Negative	Negative
Western Ridge, Nankunya	2	1623_20230510_NAN01	-23.3831990758	119.614590125	10/05/2023	5	Negative	Negative
Western Ridge, Nankunya	3	1623_20230510_NAN01	-23.3831990758	119.614590125	10/05/2023	5	Negative	Negative
								Negative

Appendix 6

eDNA Frontiers Reports

eDNA FRONTIERS | Curtin University

SERVICE REPORT

eDNA ANALYSIS

DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
NCBI	National Centre for Biotechnology Information
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
ZOTU	Zero-radius operational taxonomic unit
AIS	Alien Invasive Species
LULU	A post-clustering algorithm for curation of DNA amplicon data
18S	The nuclear gene region, 18S
COI	The mitochondrial gene region, cytochrome c oxidase I
16S	The mitochondrial subunit ribosomal RNA gene region, 16S
12S	The mitochondrial gene region, 12S

Project Details

Scope of Work: EF282

Project Title: Detection of Pilbara Olive Python and associated biodiversity of water collections taken in the Pilbara using eDNA metabarcoding

Client Details

Client: BHP Iron Ore Pty Ltd (ABN: 46 008 700 981)
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Contact: Tanya Carroll | Environmental Scientist
E: tanya.corroll@bhp.com | M: +61 419 190 573

Report Details

Report reference: EF282_BHP_RevA

Report issue date: 20/04/2023

Laboratory start date: 10/03/2023 Laboratory end date: 28/03/2023

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Approvals	Dr Kathryn Dawkins (Author)	Dr Tina Berry (Reviewer)

1.0 OBJECTIVE

The objective of this study was to assess the presence of *Liasis olivaceus barroni* (Pilbara Olive python) from water samples collected in the Pilbara region using environmental DNA (eDNA) metabarcoding.

1.1 Study Scope

Using eDNA testing, eDNA Frontiers was tasked with analysing water samples for the presence of *Liasis olivaceus barroni* (Pilbara Olive python) at several sites within the Pilbara region. The client provided a total of 36 samples consisting of water filtrate suspended on filter membranes (Tables 1 and 2). No in-field control samples were provided.

2.0 SAMPLE DETAILS

Table 1. Sample receipt details.

Date received:	22/02/2023
Transport temp:	Frozen
Number of samples:	36
Storage:	All samples were stored at -20°C prior to analysis.

Table 2. Supplied sample details.

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Filtered Date
E-282-001	eDNA.20230124.NAN01.01	Nankunya	Water	25/01/2023
E-282-002	eDNA.20230124.NAN01.02	Nankunya	Water	25/01/2023
E-282-003	eDNA.20230124.NAN01.03	Nankunya	Water	25/01/2023
E-282-004	eDNA.20230124.NAN02.01	Nankunya	Water	25/01/2023
E-282-005	eDNA.20230124.NAN02.02	Nankunya	Water	26/01/2023
E-282-006	eDNA.20230124.NAN02.03	Nankunya	Water	26/01/2023
E-282-007	1623.20230124.edna.Skel01.01	Skeleton Gorge	Water	26/01/2023
E-282-008	1623.20230124.edna.Skel01.02	Skeleton Gorge	Water	28/01/2023
E-282-009	1623.20230124.edna.Skel01.03	Skeleton Gorge	Water	28/01/2023
E-282-010	eDNA20230125.OD3.01	Opthalmia Dam	Water	26/01/2023
E-282-011	eDNA20230125.OD3.02	Opthalmia Dam	Water	26/01/2023
E-282-012	eDNA20230125.OD3.03	Opthalmia Dam	Water	26/01/2023
E-282-013	eDNA20230125.OD2.01	Opthalmia Dam	Water	26/01/2023
E-282-014	eDNA20230125.OD2.02	Opthalmia Dam	Water	26/01/2023
E-282-015	eDNA20230125.OD2.03	Opthalmia Dam	Water	26/01/2023
E-282-016	eDNA20230125.OD1.01	Opthalmia Dam	Water	26/01/2023
E-282-017	eDNA20230125.OD1.02	Opthalmia Dam	Water	26/01/2023
E-282-018	eDNA20230125.OD1.03	Opthalmia Dam	Water	26/01/2023
E-282-019	eDNA_20230125_OD07.01	Opthalmia Dam	Water	27/01/2023
E-282-020	eDNA_20230125_OD07.02	Opthalmia Dam	Water	27/01/2023
E-282-021	eDNA_20230125_OD07.03	Opthalmia Dam	Water	28/01/2023
E-282-022	eDNA20230125_OD06.01	Opthalmia Dam	Water	26/01/2023
E-282-023	eDNA20230125_OD06.02	Opthalmia Dam	Water	27/01/2023
E-282-024	eDNA20230125_OD06.03	Opthalmia Dam	Water	28/01/2023
E-282-025	eDNA20230125_OD05.01	Opthalmia Dam	Water	26/01/2023
E-282-026	eDNA20230125_OD05.02	Opthalmia Dam	Water	26/01/2023
E-282-027	eDNA20230125_OD05.03	Opthalmia Dam	Water	26/01/2023
E-282-028	eDNA.20230127.NAN01.01	Nankunya	Water	28/01/2023
E-282-029	eDNA.20230127.NAN01.02	Nankunya	Water	29/01/2023
E-282-030	eDNA.20230127.NAN01.03	Nankunya	Water	29/01/2023
E-282-031	eDNA.20230126.NAN01.01	Nankunya	Water	28/01/2023

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Filtered Date
E-282-032	eDNA.20230126.NAN01.02	Nankunya	Water	28/01/2023
E-282-033	eDNA.20230126.NAN01.03	Nankunya	Water	28/01/2023
E-282-034	eDNA.20230126.NAN01.04	Nankunya	Water	28/01/2023
E-282-035	eDNA.20230126.NAN01.05	Nankunya	Water	28/01/2023
E-282-036	eDNA.20230126.NAN01.06	Nankunya	Water	28/01/2023

3.0 METHODS

3.1 Sample Collection

Water samples were collected at 11 locations by Helix staff and filtered between the 25th and 29th January 2023. Three replicates were collected at each sampling point except 'eDNA.20230126.NAN01' where six replicates were collected. Water samples were filtered onto a filter membrane to capture eDNA present in the water. All filtering was carried out by Helix staff; no in-field control samples were supplied. Filter membranes were transported frozen to eDNA Frontiers' laboratories where they were stored at -20°C until scheduled for DNA extraction.

3.2 eDNA Extraction and Analysis

DNA was extracted from half of each filter paper using a Qiagen DNeasy blood and tissue kit, following the eDNA Frontiers lab's SOPs and detailed in Koziol *et al.,* (2018), Stat *et al.,* (2017), and Stat *et al.,* (2018). Where more than one filter paper was provided for a sample, a portion of each paper was taken to total a half filter paper. Each sample was assigned an individual combination of index tags and amplified by PCR using a 16S assay targeting reptiles. A library was generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for contamination.

3.3 Bioinformatics and Taxonomic Assignments

Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh *et al*., 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with a minimum length of 70 used. Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to OTUs, are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species level where possible. Taxonomic assignments were based on an in-house Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥95, qCov =100, LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (accessed April 2023). Additionally, Geneious Prime (version 2023.0.4) was used to align any ZOTU identified as potential *L. olivaceus barroni* against the refence sequence generated by eDNA Frontiers as it is known that there is a *L. olivaceus barroni* sequence mislabelled in the GenBank database.

It is important to note that while sequences recovered are converted to the lowest possible taxon based on similarities and differences to a DNA database (NCBI's GenBank), this database, and the taxonomic framework that underpins it, may contain errors. Accordingly, the DNA taxon identifications should be interpreted as the best available assignment based on currently available information and that errors are possible.

4.0 RESULTS

4.1 Taxonomic Diversity

Liasis olivaceus barroni was detected in a total of five samples across three sampling locations (eDNA_20230125_OD07.01, eDNA20230125_OD05.01, and eDNA.20230126.NAN01.03 through .05), with detections within a location pooled together (Table 3). The *L. olivaceus barroni* ZOTU detected in the samples matched the reference sequence generated in a previous study (100% similarity), confirming it is the target species rather than *Aspidites melanocephalus* (Black-headed Python) as indicated by GenBank.

In addition to *L. olivaceus barroni*, several species of bird, fish, mammal, reptile, and amphibian as well as some invertebrate species were detected. Taxa that had ≥95% similarity in the sequence region have been reported, with species level classification shown for matches ≥97% (Table 3). Laboratory extraction controls were all negative. As no field negative was provided, no assessment of contamination between replicates and samples can be made.

Table 3. Diversity detected from water samples using a 16S assay targeting reptiles. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (accessed April 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level; species-level taxonomy is only shown for matches ≥97%. Blue text indicates taxa whose distribution is not recorded to extend to the area according to GBIF. Blue highlighting indicates the target taxa.

Phylum	Class	Order	Family	Genus	Species	eDNA.20230124.NAN01	eDNA.20230124.NAN02	1623.20230124.edna.Skel01	eDNA20230125.OD3	eDNA20230125.OD2	eDNA20230125.OD1	eDNA_20230125_OD07	eDNA20230125_OD06	eDNA20230125_OD05	eDNA.20230127.NAN01	eDNA.20230126.NAN01
Annelida	Clitellata	Tubificida	Naididae	*Dero*							*					
Arthropoda	Insecta	Diptera	Syrphidae	*Eristalinus*	*Eristalinus punctulatus*		*									
	Ostracoda	Podocopida	Cyprididae	*Cypridopsis*	*Cypridopsis vidua*									*		
Chordata	Actinopterygii	Atheriniformes	Melanotaeniidae	*Melanotaenia*	*Melanotaenia duboulayi*	*		*	*	*	*	*	*	*		
		Cyprinodontiformes	Poeciliidae	*Poecilia*					*	*	*		*			
		Perciformes	Terapontidae	*Leiopotherapon*	*Leiopotherapon unicolor*				*	*	*		*			
	Amphibia	Anura	Myobatrachidae	*Uperoleia*										*		
			Pelodryadidae	*Ranoidea*	*Ranoidea maini*	*	*	*								
				Litoria		*	*	*		*		*	*	*	*	*
	Aves	Accipitriformes	Accipitridae	*Accipiter*			*		*						*	
		Galliformes	Phasianidae	*Gallus*							*					
		Gruiformes	Rallidae	*Porphyrio*	*Porphyrio porphyrio*		*		*		*	*		*		
		Passeriformes	Estrildidae	*Taeniopygia*	*Taeniopygia guttata*		*									
			Meliphagidae	*Ptilotula*		*		*								
			Sylviidae	*Acrocephalus*	*Acrocephalus orientalis*								*			
	Mammalia	Artiodactyla	Bovidae	*Bos*										*		*
					Bos taurus				*		*			*		
			Suidae	*Sus*	*Sus scrofa*	*										
		Diprotodontia	Macropodidae	*Macropus*	*Macropus robustus*		*									*
	-	Squamata	Elapidae	*Suta*	*Suta fasciata*		*									
			Pythonidae	*Liasis*	*Liasis olivaceus barroni*							*		*		*
	-	Testudines	Chelidae	*Chelodina*	*Chelodina steindachneri*									*		
Platyhelminthes	Catenulida	-	Stenostomidae	*Stenostomum*								*	*			
					Stenostomum cf. simplex AW-2018							*	*	*		
					Stenostomum sthenum		*				*	*	*	*		

5.0 SUMMARY

This report documents the detection of *Liasis olivaceus barroni* from environmental water samples collected from sites in the Pilbara region. The species matched with 100% similarity to the reference sequence generated in a previous study. In addition to the target taxon, several other taxonomic groups were identified. Specifically, 18 chordate genera with ≥95% similarity in the sequence region were reported, including one species whose distribution is not recorded to extend to the area. While not recorded in the area, matches to this species have occurred in many studies in the region and is likely indicative of true presence.

ARCHIVING OF STUDY DATA

The DNA extracts derived from this study will be stored within eDNA Frontiers' premises for a period of 12 months. If samples are required to be stored longer a sample archiving service can be provided.

All electronic data relating to the study is stored in an offsite secure server. This includes; all laboratory raw data; personnel records; and the study report. Hard copy documents are archived by study number into a locked area of the test facility located in eDNA Frontiers, Curtin University administration area.

REFERENCES

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Mousavi-Derazmahalleh M, Stott A, Lines R, Peverley G, Nester G, Simpson T, Zawierta M, De La Pierre M, Bunce M, Christophersen CT (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA (eDNA) sequences exploiting Nextflow and Singularity. *Molecular Ecology Resources*, 21(5), 1697-1704. https://doi.org/10.1111/1755-0998.13356.

Stat M, Huggett MJ, Bernasconi R, DiBattista JD, Berry TE, Newman SJ, Harvey ES, Bunce M (2017). Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Scientific Reports,* 7, 12240.

Stat M, John J, DiBattista JD, Newman SJ, Bunce M, Harvey ES (2018). Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology* 33(1), 196-205.

eDNA FRONTIERS | Curtin University

SERVICE REPORT

eDNA ANALYSIS

DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
NCBI	National Centre for Biotechnology Information
OTU	Operational taxonomic unit
ZOTU	Zero-radius operational taxonomic unit
AIS	Alien Invasive Species
LULU	A post-clustering algorithm for curation of DNA amplicon data
PCR	Polymerase chain reaction
mtGenome	The full mitochondrial genome
fasta	A formatting type for sequence data
18S	The nuclear gene region, 18S
COI	The mitochondrial gene region, cytochrome c oxidase I
16S	The mitochondrial subunit ribosomal RNA gene region, 16S
12S	The mitochondrial gene region, 12S

Project Details

Scope of Work: EF304

Project Title: Detection of Pilbara Olive Python and associated biodiversity of water collections taken in the Western Ridge area, Pilbara WA using eDNA metabarcoding

Client Details

Client: BHP Iron Ore Pty Ltd (ABN: 46 008 700 981)
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Report Details

Report reference: EF304_BHP_RevA

Report issue date: 14/07/2023

Laboratory start date: 27/06/2023 Laboratory end date: 05/07/2023

Test Facility

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Study Author

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Approvals	Dr Kathryn Dawkins (Author)	Dr Tina Berry (Reviewer)

1.0 OBJECTIVE

The objective of this study was to assess the presence of *Liasis olivaceus barroni* (Pilbara Olive Python) from water samples collected in the Pilbara region using environmental DNA (eDNA) metabarcoding.

1.1 Study Scope

Using eDNA testing, eDNA Frontiers was tasked with analysing water samples for the presence of *Liasis olivaceus barroni* (Pilbara Olive python) at several sites within the Pilbara region. A total of 38 samples consisting of water filtrate suspended on filter membranes were provided for analysis (Tables 1 and 2). No in-field control samples were provided.

2.0 SAMPLE DETAILS

Table 1. Sample receipt details.

Date received:	26/05/2023
Transport temp:	Frozen
Number of samples:	38
Storage:	All samples were stored at -20°C prior to analysis.

Table 2. Supplied sample details.

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Filtered Date
E-304-001	1623_20230513_NAN01_01	Nankunya	Water	13/05/2023
E-304-002	1623_20230513_NAN01_02	Nankunya	Water	13/05/2023
E-304-003	1623_20230513_NAN01_03	Nankunya	Water	13/05/2023
E-304-004	1623_20230512_NAN01_01	Nankunya	Water	12/05/2023
E-304-005	1623_20230512_NAN01_02	Nankunya	Water	12/05/2023
E-304-006	1623_20230512_NAN01_03	Nankunya	Water	12/05/2023
E-304-007	1623.20230510.Zi01_01	Zion Gorge	Water	10/05/2023
E-304-008	1623.20230510.Zi01_02	Zion Gorge	Water	10/05/2023
E-304-009	1623.20230510.Zi01_03	Zion Gorge	Water	10/05/2023
E-304-010	1623_20230510_SE01_01	Skeleton Gorge	Water	10/05/2023
E-304-011	1623_20230510_SE01_02	Skeleton Gorge	Water	10/05/2023
E-304-012	1623_20230510_SE01_03	Skeleton Gorge	Water	10/05/2023
E-304-013	1623_20230510_OPNW_01	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-014	1623_20230510_OPNW_02	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-015	1623_20230510_OPNW_03	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-016	1623_20230512_OD01_01	Ophthalmia Dam	Water	12/05/2023
E-304-017	1623_20230512_OD01_02	Ophthalmia Dam	Water	12/05/2023
E-304-018	1623_20230512_OD01_03	Ophthalmia Dam	Water	12/05/2023
E-304-019	1623_20230513_OD02_01	Ophthalmia Dam	Water	13/05/2023
E-304-020	1623_20230513_OD02_02	Ophthalmia Dam	Water	13/05/2023
E-304-021	1623_20230513_OD02_03	Ophthalmia Dam	Water	13/05/2023
E-304-022	1623_20230512_OD5_01	Ophthalmia Pool	Water	12/05/2023
E-304-023	1623_20230512_OD5_02	Ophthalmia Pool	Water	12/05/2023
E-304-024	1623_20230512_OD5_03	Ophthalmia Pool	Water	12/05/2023
E-304-025	1623_20230510_ODTypha_01	Ophthalmia Dam	Water	10/05/2023
E-304-026	1623_20230510_ODTypha_02	Ophthalmia Dam	Water	10/05/2023
E-304-027	1623_20230510_ODTypha_03	Ophthalmia Dam	Water	10/05/2023
E-304-028	1623_20230510_ODNE_01	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-029	1623_20230510_ODNE_02	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-030	1623_20230510_ODNE_03	Ophthalmia Dam, crossing	Water	10/05/2023
E-304-031	1623_20230512_OD06_01	Ophthalmia Dam	Water	12/05/2023

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Filtered Date
E-304-032	1623_20230512_OD06_02	Ophthalmia Dam	Water	12/05/2023
E-304-033	1623_20230512_OD06_03	Ophthalmia Dam	Water	12/05/2023
E-304-034	1623_20230510_NAN01_01	Nankunya	Water	10/05/2023
E-304-035	1623_20230510_NAN01_02	Nankunya	Water	10/05/2023
E-304-036	1623_20230510_NAN01_03	Nankunya	Water	10/05/2023
E-304-037	CGI_100523_A	Cathedral Gorge	Water	10/05/2023
E-304-038	CGI_100523_B	Cathedral Gorge	Water	10/05/2023

3.0 METHODS

3.1 Sample Collection

Water samples were collected at 13 sites across seven locations by Helix staff and filtered between the 10th and 13th May 2023. Three replicates were collected at each sampling point except 'CGI_100523' where two replicates were collected. Water samples were filtered onto a filter membrane to capture eDNA present in the water. All filtering was carried out by Helix staff; no in-field control samples were supplied. Half of each filter membrane was transported frozen to eDNA Frontiers' laboratories where they were stored at -20°C until scheduled for DNA extraction.

3.2 eDNA Extraction and Analysis

DNA was extracted from the supplied half-filter paper using a Qiagen DNeasy blood and tissue kit, following the eDNA Frontiers lab's Standard Operating Procedures and detailed in Koziol *et al.,* (2018), Stat *et al.,* (2017), and Stat *et al.,* (2018). Each sample was assigned an individual combination of index tags and amplified by polymerase chain reaction (PCR) using a 16S assay targeting reptiles. A library was generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for contamination.

3.3 Bioinformatics and Taxonomic Assignments

Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh *et al*., 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with a minimum length of 70 used. Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to operation taxonomic units (OTUs), are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species level where possible. Taxonomic assignments were based on an in-house Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥95, qCov =100, LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (accessed July 2023). Additionally, Geneious Prime (version 2023.1.2) was used to align any ZOTU identified as potential *L. olivaceus barroni* against the refence sequence generated by eDNA Frontiers as it is known that there is a *L. olivaceus barroni* sequence mislabelled in the GenBank database.

It is important to note that while sequences recovered are converted to the lowest possible taxon based on similarities and differences to a DNA database (NCBI's GenBank), this database, and the taxonomic framework that underpins it, may contain errors. Accordingly, the DNA taxon identifications should be interpreted as the best available assignment based on currently available information and that errors are possible.

4.0 RESULTS

4.1 Taxonomic Diversity

Liasis olivaceus barroni was detected in a total of six samples across three sampling locations (1623_20230512_NAN01_01, 1623_20230512_OD5_01, 1623_20230512_OD5_03, 1623_20230510_ODTypha_02, 1623_20230510_ODNE_03, and 1623_20230512_OD06_01), with detections within a location pooled together (Table 3). The *L. olivaceus barroni* ZOTU detected in the samples matched the reference sequence generated in a previous study (99.53-100% similarity), confirming it is the target species rather than *Aspidites melanocephalus* (Black-headed Python) as indicated by GenBank.

In addition to *L. olivaceus barroni*, several species of bird, fish, mammal, reptile, and amphibian as well as some invertebrate species were detected. Taxa that had ≥95% similarity in the sequence region have been reported, with species level classification shown for matches ≥97% (Table 3). Laboratory extraction controls were all negative. As no field negative was provided, no assessment of contamination between replicates and samples can be made.

Table 3. Diversity detected from water samples using a 16S assay targeting reptiles. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (GBIF; accessed July 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level; species-level taxonomy is only shown for matches ≥97%. Blue text indicates taxa whose distribution is not recorded to extend to the area according to GBIF. Blue highlighting indicates the target taxa.

Phylum	Class	Order	Family	Genus	Species	1623_20230513_NAN01	1623_20230512_NAN01	1623.20230510.Zi01	1623_20230510_SE01	1623_20230510_OPNW	1623_20230512_OD01	1623_20230513_OD02	1623_20230512_OD5	1623_20230510_ODTypha	1623_20230510_ODNE	1623_20230512_OD06	1623_20230510_NAN01	CGI_100523
Arthropoda	Ostracoda	Podocopida	Cyprididae	*Cypridopsis*	*Cypridopsis vidua*					*			*					
Bryozoa	Phylactolaemata	Plumatellida	Plumatellidae	*Plumatella*				*	*									
					Plumatella vaihiriae								*					
Chordata	Actinopterygii	Atheriniformes	Melanotaeniidae	*Melanotaenia*	*Melanotaenia duboulayi*					*	*	*		*	*			
		Cyprinodontiformes	Poeciliidae	*Poecilia*	*Poecilia latipinna*					*	*	*		*	*	*		
		Perciformes	Terapontidae	*Leiopotherapon*	*Leiopotherapon unicolor*	*				*	*	*		*	*	*		
	Amphibia	Anura	Pelodryadidae	*Litoria*	*Litoria rubella*	*	*	*	*				*				*	*
				Ranoidea	*Ranoidea maini*		*											*
	Aves	Accipitriformes	Accipitridae	*Accipiter*		*												
		Anseriformes	Anatidae	*Anas*	*Anas platyrhynchos*							*						
				Cygnus	*Cygnus atratus*								*					
				Tadorna	*Tadorna ferruginea*						*			*				
		Galliformes	Phasianidae	*Gallus*	*Gallus gallus*					*								
		Gruiformes	Rallidae	*Fulica*	*Fulica atra*						*			*				
				Porphyrio	*Porphyrio porphyrio*					*		*						
		Passeriformes	Estrildidae	*Taeniopygia*	*Taeniopygia guttata*	*	*		*								*	
			Meliphagidae	*Ptilotula*	*Ptilotula penicillata*				*							*		
			Monarchidae	*Grallina*	*Grallina cyanoleuca*										*			
			Pachycephalidae	*Colluricincla*	*Colluricincla harmonica*			*										
			Ptilonorhynchidae	*Ptilonorhynchus*	*Ptilonorhynchus violaceus*		*										*	
			Rhipiduridae	*Rhipidura*				*			*							
			Sylviidae	*Acrocephalus*								*						
		Pelecaniformes	Ardeidae	*Egretta*	*Egretta novaehollandiae*						*							
		Podicipediformes	Podicipedidae	*Tachybaptus*	*Tachybaptus novaehollandiae*								*					
		Psittaciformes	Psittaculidae	*Melopsittacus*	*Melopsittacus undulatus*		*											
	Mammalia	Artiodactyla	Bovidae	*Bos*							*		*			*		*
			Suidae	*Sus*	*Sus scrofa*	*												*
		Carnivora	Canidae	*Canis*	*Canis lupus familiaris*	*				*					*			
		Chiroptera	Emballonuridae	*Saccolaimus*	*Saccolaimus flaviventris*										*			
			Vespertilionidae	*Chalinolobus*	*Chalinolobus gouldii*	*												
	-	Squamata	Pythonidae	*Liasis*	*Liasis olivaceus barroni*		*						*	*	*	*		
	-	Testudines	Chelidae	*Chelodina*	*Chelodina steindachneri*											*		
Platyhelminthes	Catenulida	-	Stenostomidae	*Stenostomum*	*Stenostomum cf. simplex AW-2018*	*					*	*	*		*	*		
					Stenostomum sthenum								*			*		

5.0 SUMMARY

This report documents the detection of *Liasis olivaceus barroni* from environmental water samples collected from sites in the Pilbara region. The ZOTUs detected matched with 99.53-100% similarity to the reference sequence generated for this species in a previous study. In addition to the target taxon, several other taxonomic groups were identified. Specifically, 32 chordate genera with ≥95% similarity in the sequence region were recorded, including three species whose distribution is not recorded to extend to the area. These non-endemic detections were matched to GenBank reference sequences at ≥97% sequence similarity; it is possible that these ZOTUs represent other closely related species from the area that do not have reference sequences available, or they are true detections of these organisms.

ARCHIVING OF STUDY DATA

The DNA extracts derived from this study will be stored within eDNA Frontiers' premises for a period of 12 months. If samples are required to be stored longer a sample archiving service can be provided.

All electronic data relating to the study is stored in an offsite secure server. This includes; all laboratory raw data; personnel records; and the study report. Hard copy documents are archived by study number into a locked area of the test facility located in eDNA Frontiers, Curtin University administration area.

REFERENCES

Edgar RC (2018). Updating the 97% identity threshold for 16S ribosomal RNA OTUs. *Bioinformatics* 34(14), 2371-2376.

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Mousavi-Derazmahalleh M, Stott A, Lines R, Peverley G, Nester G, Simpson T, Zawierta M, De La Pierre M, Bunce M, Christophersen CT (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA (eDNA) sequences exploiting Nextflow and Singularity. *Molecular Ecology Resources*, 21(5), 1697-1704. https://doi.org/10.1111/1755-0998.13356.

Stat M, Huggett MJ, Bernasconi R, DiBattista JD, Berry TE, Newman SJ, Harvey ES, Bunce M (2017). Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Scientific Reports,* 7, 12240.

Stat M, John J, DiBattista JD, Newman SJ, Bunce M, Harvey ES (2018). Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology* 33(1), 196-205.

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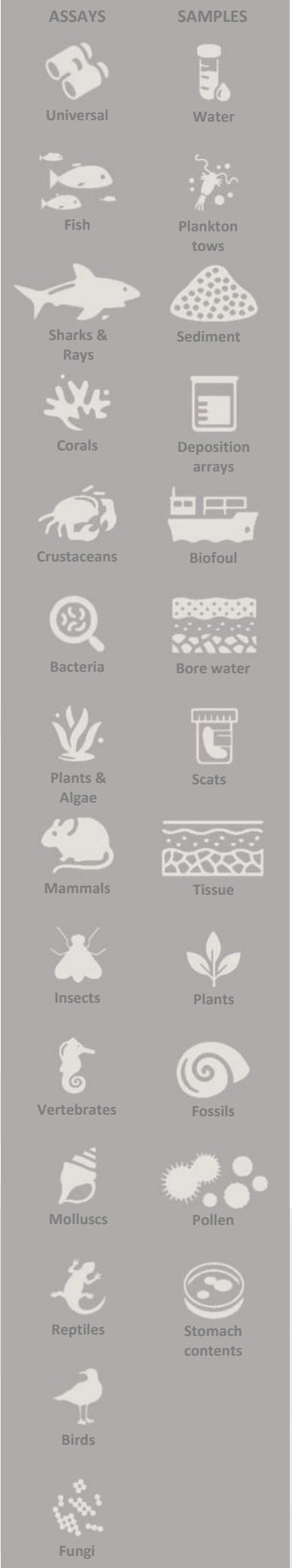
SERVICE REPORT

eDNA ANALYSIS



DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
NCBI	National Centre for Biotechnology Information
OTU	Operational taxonomic unit
ZOTU	Zero-radius operational taxonomic unit
AIS	Alien Invasive Species
LULU	A post-clustering algorithm for curation of DNA amplicon data
PCR	Polymerase chain reaction
mtGenome	The full mitochondrial genome
fasta	A formatting type for sequence data
18S	The nuclear gene region, 18S
COI	The mitochondrial gene region, cytochrome c oxidase I
16S	The mitochondrial subunit ribosomal RNA gene region, 16S
12S	The mitochondrial gene region, 12S

Project Details

Scope of Work: EF317

Project Title: Detection of Pilbara Olive Python and associated biodiversity of water collections taken from Millstream, Pilbara WA using eDNA metabarcoding

Client Details

Client: BHP Iron Ore Pty Ltd (ABN: 46 008 700 981)
Brookfield Place, 125 St Georges Terrace, Perth WA 6000

Contact: Tanya Carroll | Environmental Scientist
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Report Details

Report reference: EF317_BHP_RevA

Report issue date: 14/07/2023

Laboratory start date: 27/06/2023 Laboratory end date: 05/07/2023

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Approvals	Dr Kathryn Dawkins (Author)	Georgia Peverley (Reviewer)

1.0 OBJECTIVE

The objective of this study was to assess the presence of *Liasis olivaceus barroni* (Pilbara Olive Python) from water samples collected in the Pilbara region using environmental DNA (eDNA) metabarcoding.

1.1 Study Scope

Using eDNA testing, eDNA Frontiers was tasked with analysing water samples for the presence of *Liasis olivaceus barroni* (Pilbara Olive python) at several sites within the Pilbara region. A total of 15 samples consisting of water filtrate suspended on filter membranes were provided for analysis (Tables 1 and 2). No in-field control samples were provided.

2.0 SAMPLE DETAILS

Table 1. Sample receipt details.

Date received:	16/06/2023
Transport temp:	Frozen
Number of samples:	15
Storage:	All samples were stored at -20°C prior to analysis.

Table 2. Supplied sample details.

eDNA Frontiers ID	Client Sample ID	Collection Location	Sample Type	Filtered Date
E-317-001	1623-20230525_millstream_zh05_01	Millstream	Water	25/05/2023
E-317-002	1623-20230525_millstream_zh05_02	Millstream	Water	25/05/2023
E-317-003	1623-20230525_millstream_zh05_03	Millstream	Water	25/05/2023
E-317-004	1623_20230525_millstream_zh06_01	Millstream	Water	25/05/2023
E-317-005	1623_20230525_millstream_zh06_02	Millstream	Water	25/05/2023
E-317-006	1623_20230525_millstream_zh06_03	Millstream	Water	25/05/2023
E-317-007	1623_20230523_Millstream_ZH03_01	Millstream	Water	23/05/2023
E-317-008	1623_20230523_Millstream_ZH03_02	Millstream	Water	23/05/2023
E-317-009	1623_20230523_Millstream_ZH03_03	Millstream	Water	23/05/2023
E-317-010	1623-20230523-millstream-zh01_01	Millstream	Water	23/05/2023
E-317-011	1623-20230523-millstream-zh01_02	Millstream	Water	23/05/2023
E-317-012	1623-20230523-millstream-zh01_03	Millstream	Water	23/05/2023
E-317-013	1623_20230523_MillstreamZH02_01	Millstream	Water	23/05/2023
E-317-014	1623_20230523_MillstreamZH02_02	Millstream	Water	23/05/2023
E-317-015	1623_20230523_MillstreamZH02_03	Millstream	Water	23/05/2023

3.0 METHODS

3.1 Sample Collection

Water samples were collected at five locations by Helix staff, with three replicates collected at each sampling point. Water samples were filtered onto a filter membrane to capture eDNA present in the water. All filtering was carried out by Helix staff between the 23rd and 25th May; no in-field control samples were supplied. Half of each filter membrane was transported frozen to eDNA Frontiers' laboratories where they were stored at -20°C until scheduled for DNA extraction.

3.2 eDNA Extraction and Analysis

DNA was extracted from the supplied half-filter paper using a Qiagen DNeasy blood and tissue kit, following the eDNA Frontiers lab's Standard Operating Procedures and detailed in Koziol *et al.,* (2018), Stat *et al.,* (2017), and Stat *et al.,* (2018). Each sample was assigned an individual combination of index tags and amplified by polymerase chain reaction (PCR) using a 16S assay targeting reptiles. A library was generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for contamination.

3.3 Bioinformatics and Taxonomic Assignments

Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh *et al*., 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with a minimum length of 70 used. Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to operation taxonomic units (OTUs), are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species level where possible. Taxonomic assignments were based on an in-house Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥95, qCov =100, LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (accessed July 2023).

It is important to note that while sequences recovered are converted to the lowest possible taxon based on similarities and differences to a DNA database (NCBI's GenBank), this database, and the taxonomic framework that underpins it, may contain errors. Accordingly, the DNA taxon identifications should be interpreted as the best available assignment based on currently available information and that errors are possible.

4.0 RESULTS

4.1 Taxonomic Diversity

Liasis olivaceus barroni was not detected in any sample across the five locations (Table 3). However, several species of bird, fish, and mammal as well as one invertebrate were detected. Taxa that had ≥95% similarity in the sequence region have been reported, with species level classification shown for matches ≥97% (Table 3). Laboratory extraction controls were all negative. As no field negative was provided, no assessment of contamination between replicates and samples can be made.

Table 3. Diversity detected from water samples using a 16S assay targeting reptiles. Presence of the species at each site is indicated by the symbol *. Taxonomy was assigned as per NCBI and classifications were standardised according to the Global Biodiversity Information Facility (GBIF; accessed July 2023). Blank cells indicate where taxa could not be resolved to a lower taxonomic level; species-level taxonomy is only shown for matches ≥97%. Blue text indicates taxa whose distribution is not recorded to extend to the area according to GBIF. Blue highlighting indicates the target taxa.

Phylum	Class	Order	Family	Genus	Species	1623-20230525_millstream_zh05	1623_20230525_millstream_zh06	1623_20230523_Millstream_ZH03	1623-20230523-millstream-zh01	1623_20230523_MillstreamZH02
Bryozoa	Phylactolaemata	Plumatellida	Plumatellidae	*Plumatella*					*	
Chordata	Actinopterygii	Atheriniformes	Melanotaeniidae	*Melanotaenia*	*Melanotaenia duboulayi*	*	*	*	*	*
		Clupeiformes	Clupeidae	*Nematalosa*	*Nematalosa erebi*	*		*	*	*
		Perciformes	Gobiidae	*Glossogobius*	*Glossogobius aureus*	*		*	*	*
			Terapontidae	*Leiopotherapon*	*Leiopotherapon aheneus*	*		*	*	*
					Leiopotherapon unicolor	*		*	*	
		Siluriformes	Ariidae					*		
	Aves	Anseriformes	Anatidae							*
		Galliformes	Phasianidae	*Gallus*	*Gallus gallus*	*			*	*
		Gruiformes	Rallidae	*Porphyrio*	*Porphyrio porphyrio*	*				
		Passeriformes	Estrildidae	*Taeniopygia*		*				
	Mammalia	Artiodactyla	Bovidae	*Bos*	*Bos taurus*	*			*	
			Suidae	*Sus*	*Sus scrofa*			*	*	

5.0 SUMMARY

This analysis did not detect the target species *Liasis olivaceus barroni* from any environmental water sample collected from sites in the Pilbara region. However, several other taxonomic groups were identified. Specifically, nine chordate genera with ≥95% similarity in the sequence region were recorded, including two species whose distribution is not recorded to extend to the area. These non-endemic detections were matched to GenBank reference sequences at ≥97% sequence similarity; it is possible that these ZOTUs represent other closely related species from the area that do not have reference sequences available, or they are true detections of these organisms.

ARCHIVING OF STUDY DATA

The DNA extracts derived from this study will be stored within eDNA Frontiers' premises for a period of 12 months. If samples are required to be stored longer a sample archiving service can be provided.

All electronic data relating to the study is stored in an offsite secure server. This includes; all laboratory raw data; personnel records; and the study report. Hard copy documents are archived by study number into a locked area of the test facility located in eDNA Frontiers, Curtin University administration area.

REFERENCES

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Global Biodiversity Information Facility. https://www.gbif.org/ (accessed July 2023).

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Mousavi-Derazmahalleh M, Stott A, Lines R, Peverley G, Nester G, Simpson T, Zawierta M, De La Pierre M, Bunce M, Christophersen CT (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA (eDNA) sequences exploiting Nextflow and Singularity. *Molecular Ecology Resources*, 21(5), 1697-1704. https://doi.org/10.1111/1755-0998.13356.

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Stat M, John J, DiBattista JD, Newman SJ, Bunce M, Harvey ES (2018). Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology* 33(1), 196-205.

Appendix 7

Pilbara Olive Python Radio-tracking Summaries

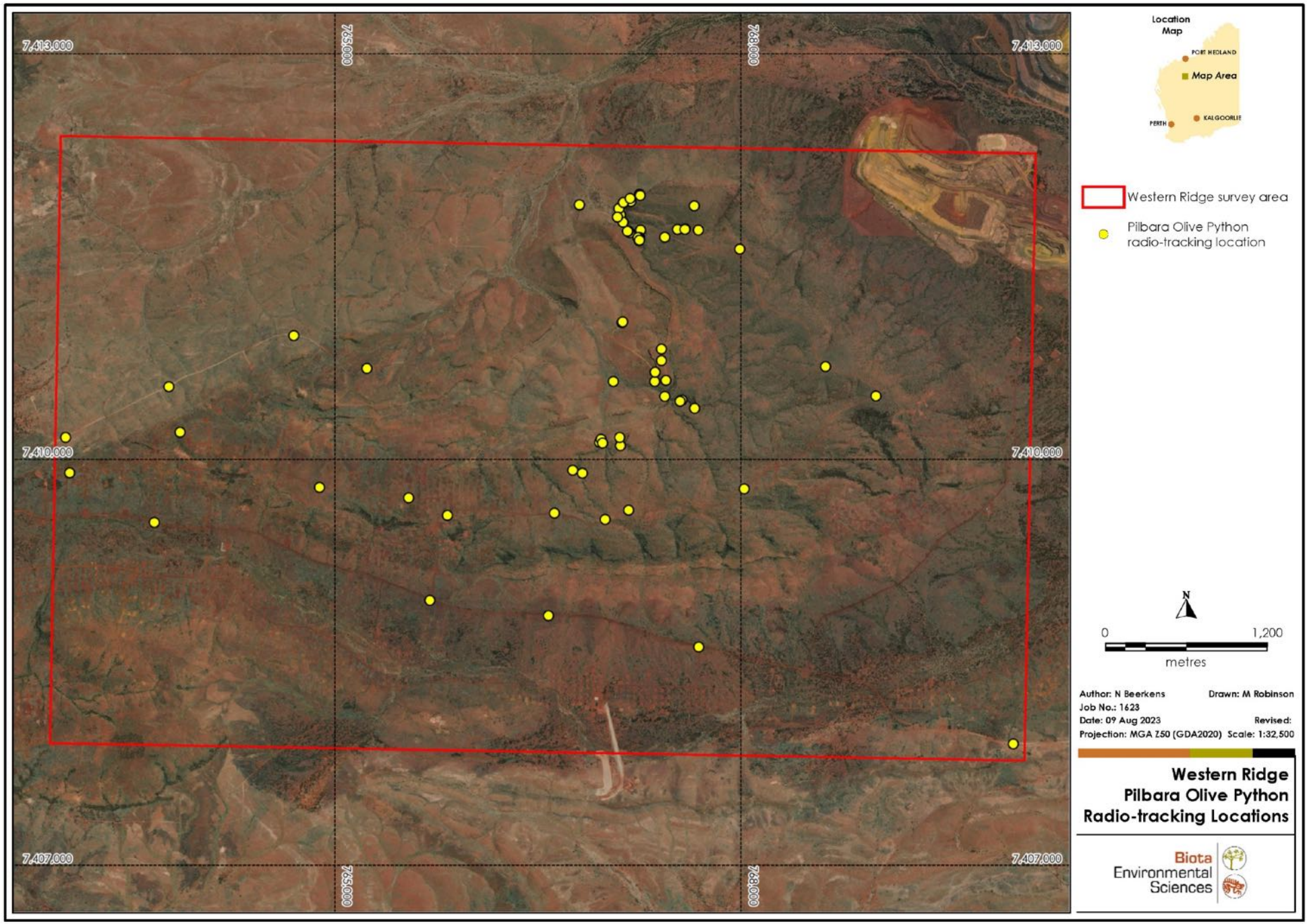


Figure Error! No text of specified style in document.**.1: Locations where radiotracking was attempted to detect signals of Pilbara Olive Python at Western Ridge.**

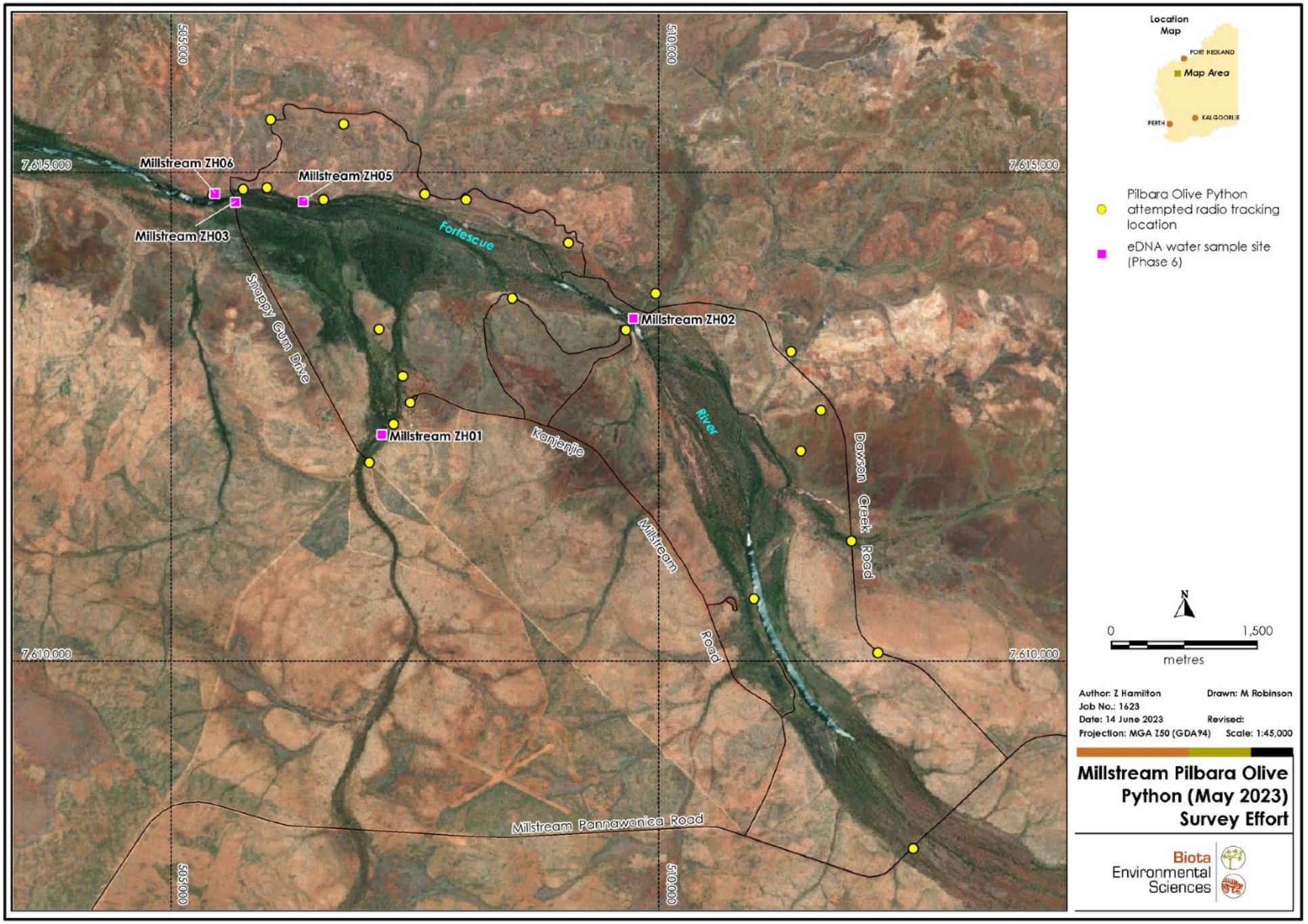
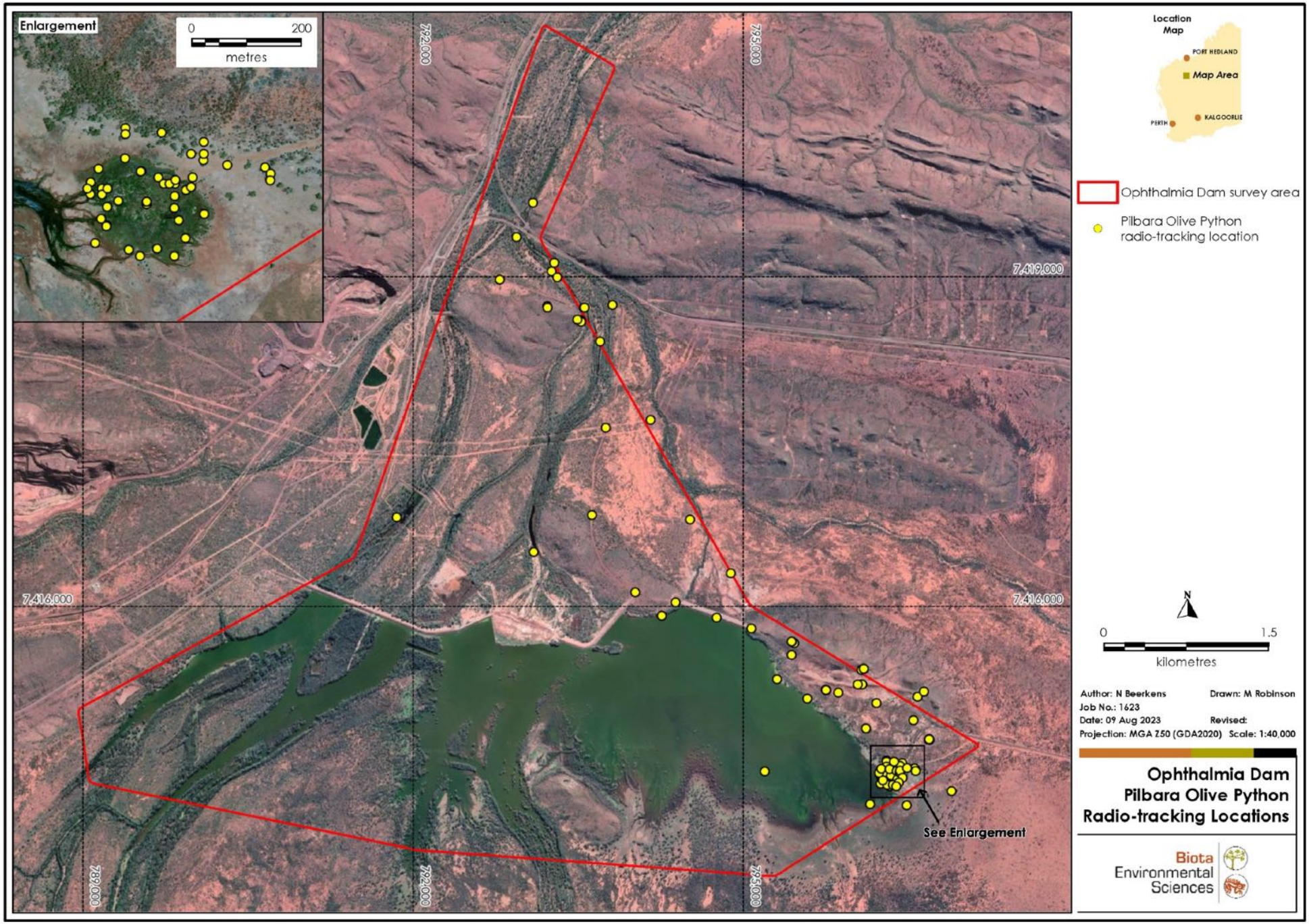


Figure Error! No text of specified style in document.**.2. Locations where radiotracking was attempted to detect signals of Pilbara Olive Python at Millstream-Chichester National Park.**


Enlargement
0
200
metres
792,000
796,000
7,419,000
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See Enlargement
Location Map
PORT HEDLAND
Map Area
PERTH
KALGOORLIE
Ophthalmia Dam survey area
Pilbara Olive Python radio-tracking location
N
0
1.5
kilometres
Author: N Beerkens
Drawn: M Robinson
Job No.: 1623
Date: 09 Aug 2023
Revised:
Projection: MGA Z50 (GDA2020)
Scale: 1:40,000
Ophthalmia Dam
Pilbara Olive Python
Radio-tracking Locations
Biota
Environmental
Sciences

Figure Error! No text of specified style in document..**3.** **Locations where radiotracking was attempted to detect signals of Pilbara Olive Python at Ophthalmia Dam.**

1.1 Western Ridge

POP 201, a sub-adult female, was originally captured and released in Phase 1 in Xanadu Gorge (16 January 2022). During phase 2, we attempted to track her on five nights, but received no signal until the fifth and final night (1 March 2022). That night, she was relocated in a small breakaway north of the entrance to Xanadu Gorge, 240 m from her release point. In September 2022, no signal was detected during tracking attempts by BHP staff Dr Matt Love and Jared Leigh. In December 2022, phase 3 of the Biota survey, her signal was detected twice from two attempts, firstly on 7 December 2022, and then again on 9 December 2022. Due to time constraints, she was not tracked to a location on 7 December. On 9 December, she was tracked to a narrow, dry gorge ("East Skeleton Gorge"), where the signal emanated strongly from an inaccessible cluster of boulders and crevices on the gorge wall, 540m SW of her last known location. Although the python was not seen, we are confident that we were within 10m of it.

In Phase 4 of the Biota survey, POP 201 was successfully radio-tracked three times from three attempts and observed on two of those occasions (see Table 1, Figure **Error! No text of specified style in document.**.4). Firstly, on 24 January 2023, she was found basking on a rock outcrop at the entrance to West Skeleton Gorge, 150m W of her last known location (see Plate 1). She was promptly hand-captured, weighed, given a health assessment, and then released. She was in excellent condition, and at 1,210g, had put on 305g since her initial capture. Her microchip scanned properly, and surgical scar had healed very well, to the point of being almost invisible (see Plate 2). On January 26 2023, she was tracked into the southern branch of West Skeleton Gorge, approximately 270m straight-line distance from the previous location (or ~350m away, if you follow the shape of the gorge). The direction of VHF-signals indicated that it was certainly in this southern branch, however, its exact location was inaccessible. As such, the GPS location provided is an estimate, based on signal strength and direction, considered accurate to within 80m. The following day, January 27, 2023, the animal was tracked to 25 m downhill of its position of January 24. It was found stationary beneath small, sparse spinifex (Plate 3). As it appeared healthy and had received a health assessment just three days prior, it was not handled. In phase 5 (9-14 May 2023), it was tracked on each day and/or night of the Western Ridge survey, and no signal was detected despite extensive searching.

Plate 1. **POP 201, as found on 24/01/2023, one year after being fitted with a VHF transmitter. Healthy and basking on a rock outcrop.**

Plate 2. **POP 201's surgical scar on 24/01/2023; barely visible.**

Plate 3. **POP 201, as found on 27/01/2023.**

Table 1. **Known locations of POP 201.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
16/01/2022	Xanadu gorge	-23.3939	119.6179	+/- 2m	Dry, rocky gorge floor. Initial capture.
1/03/2022	Xanadu gorge entrance	-23.3927	119.6159	+/- 5m	In small rocky breakaway. Animal not observed.
9/12/2022	East Skeleton gorge	-23.3970	119.6135	+/- 10m	Up dry gorge wall, amongst inaccessible boulders and crevices. Animal not observed.
24/01/2023	West Skeleton gorge entrance	-23.3968	119.6120	+/- 2m	Found basking on a rock outcrop, up the entrance to West Skeleton gorge. Animal recaptured, given a health assessment and released.
26/01/2023	West Skeleton gorge	-23.3989	119.6108	+/- 80m	In southern branch of West Skeleton gorge; animal not observed. Location estimated based on signal strength and direction.
27/01/2023	West Skeleton gorge entrance	-23.3966	119.6121	+/- 2m	Found stationary at the entrance to West Skeleton gorge; 25m downhill of its location on 24/01/2023. Animal appeared healthy and was left alone.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

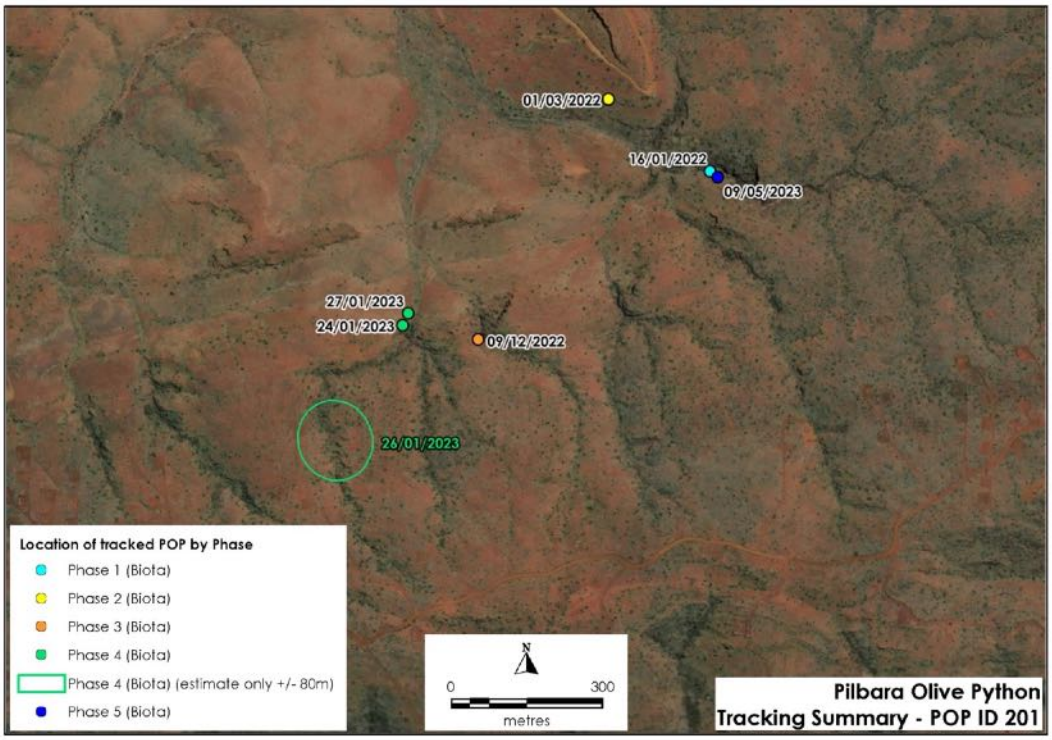

Figure Error! No text of specified style in document.**.4.** **POP 201 radio-tracked locations at Western Ridge, 2022 and 2023.**

POP 202, an adult female, was captured in Phase 2 of this survey, on 24 February 2022 in the gorge at Nankunya (Afghan Spring) (see Table 2) and represents a long-term recapture event for the area. Previously, she had been captured and microchipped by Biologic in 2020, 156m from her where we first observed her. One attempt was made during phase 2 to relocate her, and she was tracked to beneath a rock, within dense reeds, alongside a shallow stream, <5 m from her release site Plate 4). Despite this proximity to her release point, she was so well hidden that she would not have been found if not for radiotracking. On 8 September 2022, during a radio-tracking session by BHP staff Dr Matt Love and Jared Leigh, POP 202 was found deceased beneath a relatively expansive rocky crevice, ~10m away from the main Nankunya spring (see Plate 5 (see Figure **Error! No text of specified style in document.**.5). The body was headless and decaying, estimated to have died in the preceding two-three weeks. Dingoes, which were regularly recorded on camera traps set in Nankunya between Phases 3 and 4, may be responsible for killing this large python.

Plate 4. **POP 202, as found on 1/03/2022 – coiled beneath a rock behind dense reeds at the edge of a shallow stream.**

Plate 5. **Two images of the decomposing body, as found on 8 September 2022. Photos supplied by Dr Matt Love of BHP.**

Table 2. Recorded locations of POP 202.

Date	Location	Latitude	Longitude	Accuracy*	Comments
15/03/2020	Nankunya	-23.3821	119.6134	-	First known record of this individual (Biologic 2021).
24/02/2022	Nankunya	-23.3831	119.6145	+/- 2m	In a dry creekbed, 5m from a shallow pond. Initial capture for this project.
1/03/2022	Nankunya	-23.3831	119.6145	+/- 2m	Found coiled under rock within dense reeds alongside a shallow stream. Appeared healthy.
08/09/2022	Nankunya	-23.3831	119.6145	+/- 2m	Found deceased and headless beneath a relatively expansive rocky crevice, ~10m from the main spring. Decaying, and estimated to have died in the preceding two-three weeks.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

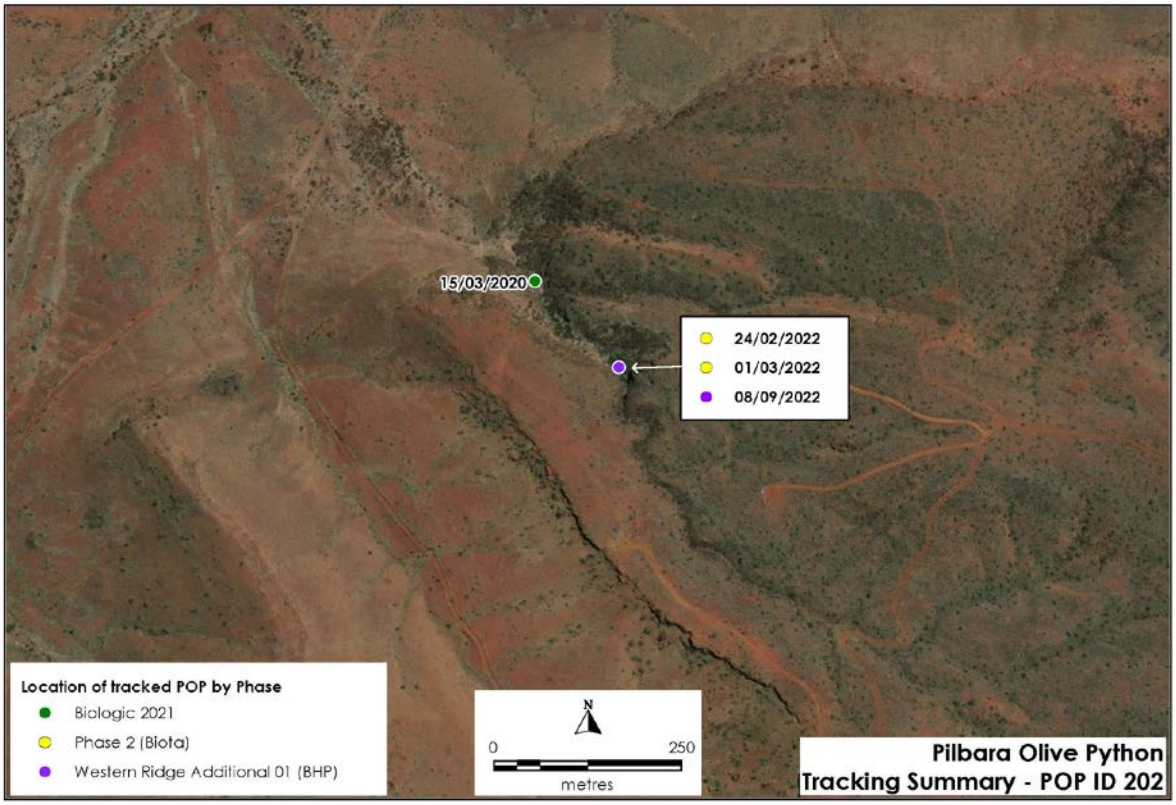

Figure Error! No text of specified style in document.**.5. Records of radio-signal tracking location for POP 202, 2022 and 2023. Original capture location, 2020, also indicated.**

POP 211, an adult male, was captured in phase 3 of this survey, on 8 December 2022 in the main spring at Nankunya. Despite tracking attempts across Western Ridge and Nankunya on all three nights of the phase 4 Western Ridge survey (24, 26 and 27 January 2023), no signals of the animal were detected (Table 3, Figure **Error! No text of specified style in document.**.6). In May 2023, it was tracked on each day and/or night of the Western Ridge survey (9-14 May). Its signal was only detected once, on the night of 12 May, at which time it was found coiled beneath NW-facing rocks along a ridgeline and appeared healthy (see Plate 6, Plate 7). After that, its signal could no longer be found, and it was no longer visible under those same rocks. It likely moved deeper into an ironstone crevice.

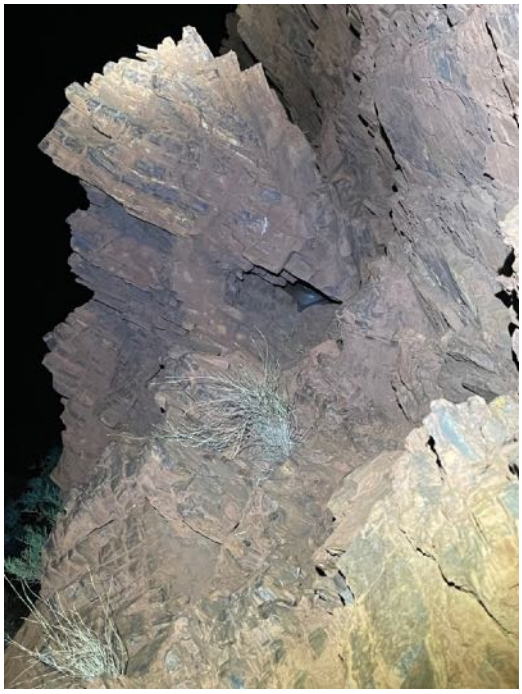

Plate 6. POP 211 as found on 12/05/2023, beneath ironstone rocks, the NW aspect of a ridgeline.

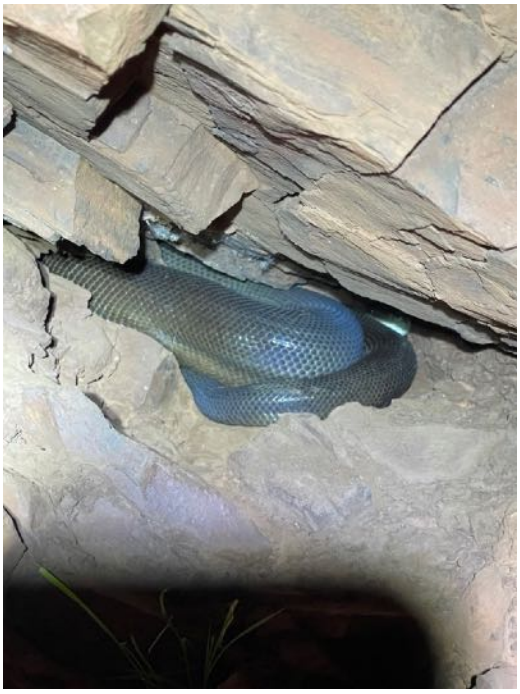

Plate 7. POP 211 as found on 12/05/2023 – closer image.

Table 3. Known locations of POP 211.

Date	Location	Latitude	Longitude	Accuracy*	Comments
8/12/2022	Nankunya	-23.3833	119.6146	+/- 2m	Underwater, in the main spring at Nankunya. Initial capture.
12/05/2023	NE of Nankunya	-23.3825	119.6179	+/- 2m	Found coiled beneath NW-facing rocks, along a ridgeline. Appeared healthy.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

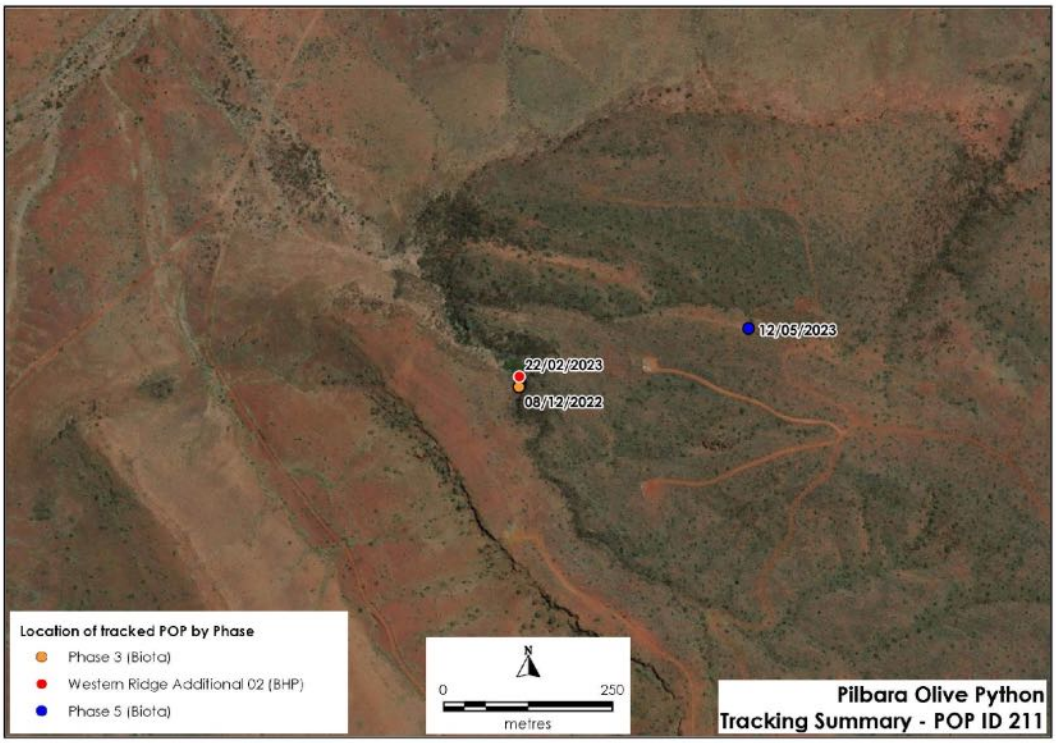

Figure Error! No text of specified style in document.**.6. Records of radio-signal tracking and capture locations for POP 211, 2022 and 2023.**

POP 212, an adult female, was captured in phase 3 of this survey, on 8 December 2022 moving down the gorge edge towards the reed-covered stream at the base of Nankunya gorge. Despite tracking attempts across Western Ridge and Nankunya on all three nights of the phase 4 and 5 Western Ridge surveys (24, 26 and 27 January and 9-14 May 2023), no signals of the animal were detected (Table 4, Figure **Error! No text of specified style in document.**.7). This may indicate that it has moved a significant distance away, was deep within an ironstone crevice, or both.

Table 4. Known locations of POP 212.

Date	Location	Latitude	Longitude	Accuracy*	Comments
8/12/2022	Nankunya	-23.3831	119.6146	+/- 2m	Moving down the gorge edge, towards the reed-covered stream. Initial capture.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

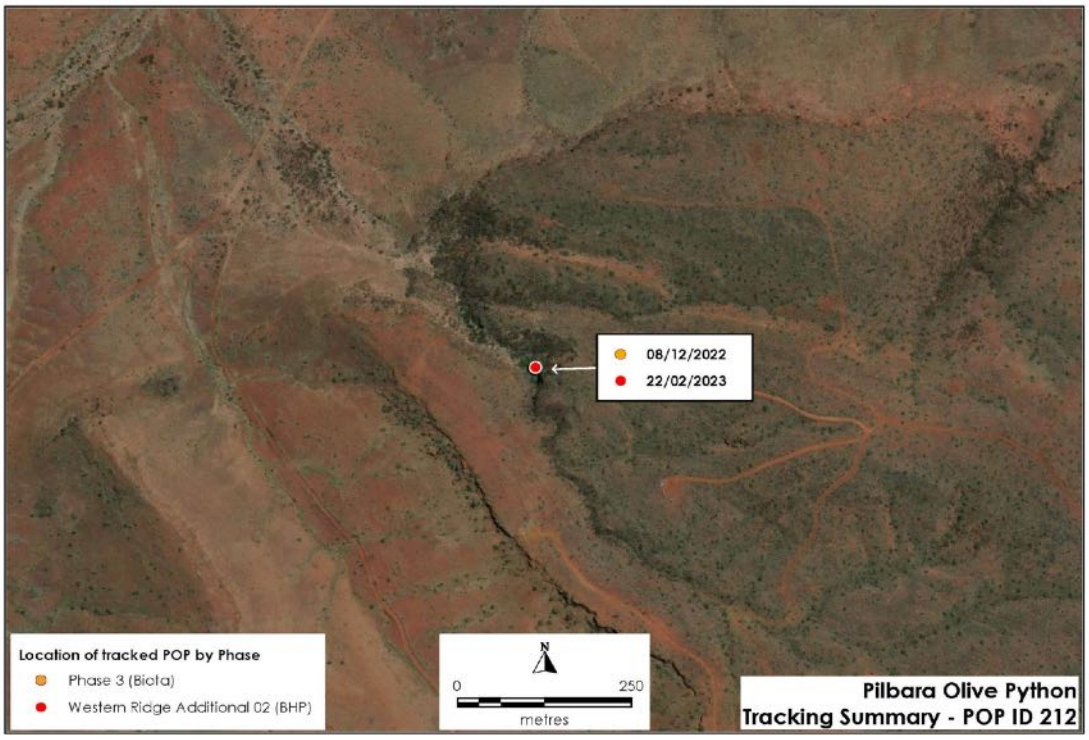

Figure Error! No text of specified style in document..**7. Records of radio-signal tracking and capture location for POP 212, 2022 and 2023.**

POP 216, an adult female, was first captured for this project in phase 4, on 26 January 2023 (see Figure **Error! No text of specified style in document.**.8, Table 5) and represents a long-term recapture for the site - having been microchipped and observed twice by Biologic in 2020 (microchip 900193003604460; Biologic 2021). Unfortunately, the first of the two GPS locations provided for the 2020 observations appears to be erroneous, being located in Cathedral Gorge, rather than Nankunya (Biologic 2021). The report's text confirms that both sightings of the animal were at Nankunya (under the location name "VWER-10"; Biologic 2021). The second Biologic observation point appears more reliable, being 90 m from our capture location. During this second observation, with the POP weighed 3,355g (Biologic 2021). Upon our first capture, it weighed 2,750g, a loss of 605g. The python was fitted with a VHF transmitter on 27/01/2023, released at point of capture. In Phase 5, it was tracked on each day and/or night of the Western Ridge survey (9-14 May 2023), and its signal was detected daily (see Table 5). On the nights of 9 and 10 May, it was found beneath a single isolated rock on a W aspect slope N of the entrance to Nankunya (Plate 8). Over the following four nights, it progressively moved N/NE, following the ridgeline's NW aspect edge, and by 14 May was ~230 m from its 9 May location, in a 5cm wide crevice at the base of a N-facing ironstone ridge (see Plate 10, Plate 11, Plate 12 and Plate 13). It was sighted during each tracking attempt, except for 14 May, and appeared healthy each time, with surgical wounds that were healing well (Plate 9).

Plate 8. The isolated rock which POP 216 was found under on 9 and 10/05/2023, on a W aspect slope.

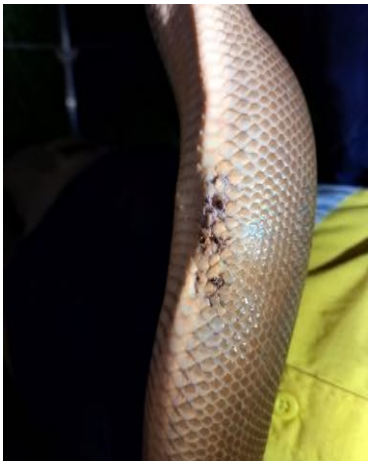

Plate 9. Surgical scar of POP 216, as found on 11/05/2023, ~4 months post-surgery. Healing well.

Plate 10. **POP 216, as found on 11/05/2023.**

Plate 11. **POP 216, as found on 12/05/2023.**

Plate 12. **POP 216, as found on 13/05/2023.**

Plate 13. **The 5cm wide crevice which POP 216 was tracked to on 14/05/2023, at the base of a N-aspect ridgeline.**

Table 5. **Known locations of POP 216.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
27/08/2020	Nankunya	-23.2887 (?)	119.7466 (?)	Likely erroneous	From Biologic 2021. The report's text confirms that this microchipped python was observed twice in Nankunya in 2020 (under the location name "VWER-10"). However, this GPS location is from Cathedral Gorge, and therefore is likely erroneous.
26/11/2020	Nankunya	-23.3828	119.6139	-	From Biologic 2021.
26/01/2023	Nankunya	-23.3827	119.6138	+/- 2m	In dry streambed beside reeds. Initial capture for this project.
09/05/2023	N of Nankunya	-23.3816	119.6132	+/- 2m	Observed beneath a standalone rock on a NW aspect slope north of Nankunya. Appeared healthy.
10/05/2023	N of Nankunya	-23.3816	119.6132	+/- 2m	Same place and position as yesterday. Appeared healthy.
11/05/2023	N of Nankunya	-23.3812	119.6131	+/- 2m	Found stretched out on W aspect BIF outcrop, 20 m N of its previous location. Hand-captured for health assessment (healthy with scar healing well), and immediately re-released at point of capture.
12/05/2023	N of Nankunya	-23.3808	119.6134	+/- 2m	Found half-emerged from rocks on NW aspect BIF outcrop, 70 m NE of its previous location. Appeared healthy.
13/05/2023	N of Nankunya	-23.3805	119.6139	+/- 2m	Found stretched out on NW aspect BIF outcrop, 60 m NE of its previous location. Appeared healthy.
14/05/2023 1630 hrs	N of Nankunya	-23.3803	119.6146	+/- 2m	Located (but not observed) in 4cm wide hole extending into base of NW aspect BIF outcrop, 80 m NE of its previous location.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

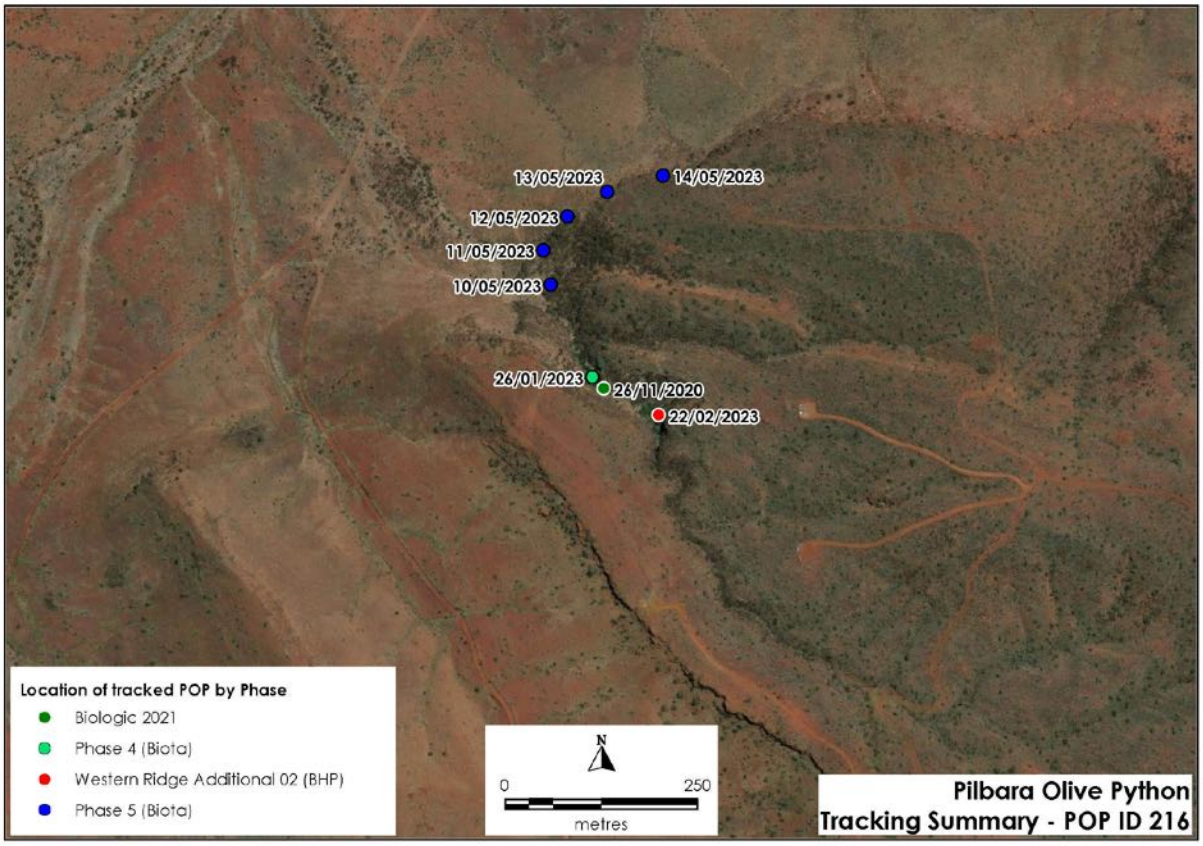

Figure Error! No text of specified style in document..**8. POP 216 capture location at Western Ridge during phase 4 survey (2023) and previous record of individual from 2020 (Biologic 2021).**

1.2 Millstream

POP 101, an adult male, was first captured on 11/01/22, during phase 1 in a small creekline west of the Millstream homestead. It was fitted with a 28g AI-T2 VHF transmitter on 12/01/22 and released at point of capture that night. No signal was detected by DBCA's Dr. David Pearson while radio-tracking across Millstream in either June 2022 or October 2022. On 25/10/22, its' signal was detected by BHP staff Dr. Matt Love, Jared Leigh, Tanya Carroll and Suzi Wild, and tracked to thick vegetation along a flowing creekline 70 m N of its previous location. The following morning, 26/10/22, the snake was tracked again to the same location (see Table 6). In both cases, the snake was not observed, but its location was deduced by two tracking teams. In May 2023, no signal was detected by Biota staff, despite extensive tracking throughout the Millstream study area (Figure **Error! No text of specified style in document.**.9).

Table 6. Known locations of POP 101.

Date	Location	Latitude	Longitude	Accuracy*	Comments
11/01/2022	Creekline W of Millstream homestead	-21.5894	117.0708	+/- 2m	Found in a flowing creekline, between a 3m wide gap in Typha. Initial capture.
25/10/2022	Creekline W of Millstream homestead	-21.5888	117.0709	+/- 10m	In thick vegetation within flowing creek bed, 70m N of previous location. Not observed.
26/10/2022	Creekline W of Millstream homestead	-21.5887	117.0709	+/- 10m	In same location as previous. Not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

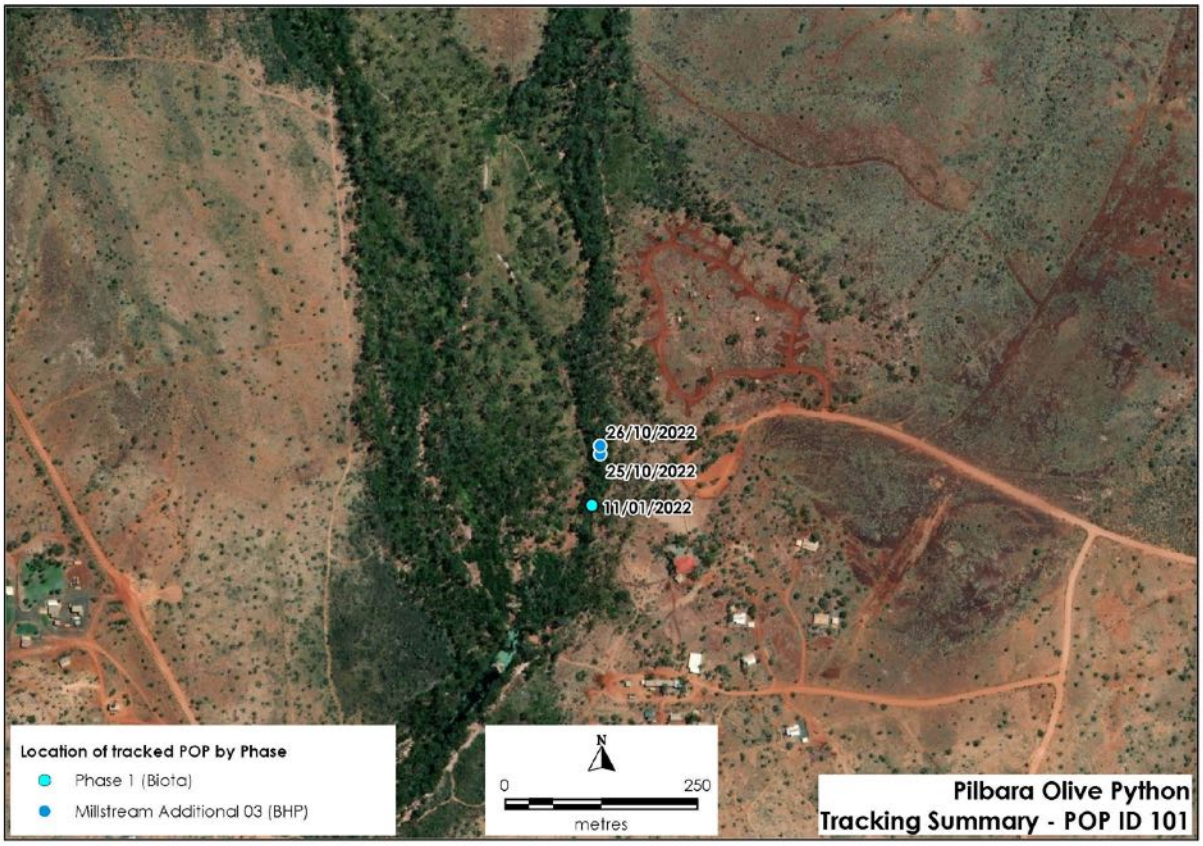

Figure Error! No text of specified style in document..**9. Records of radio-signal tracking locations for POP 101 at Millstream.**

POP 102, a sub-adult female, was first captured on 11/01/22, during phase 1 east of Palm Crossing, at the base of an ironstone ridgeline, moving into a Typha reedbed. It was fitted with a 28g AI-T2 VHF transmitter and released on 12/01/22 at point of capture. On 13/01/22, it was relocated within a heavily vegetated creekline ~100m east of its previous location. Across six days in June and August 2022, it was tracked to a single point on an ironstone ridgeline west of the track by DBCA's Dr. David Pearson while radio-tracking across Millstream in either June 2022 or October 2022. On 26/10/22, it was tracked by BHP staff Dr. Matt Love, Jared Leigh, Tanya Carroll and Suzi Wild, to an ironstone crevice 160m S of its previous location. It was in the same crevice as male POP 103, but neither were sighted. In phase 6, on the morning of 26/05/2023, its' location was triangulated in a Typha reedbed, in the centre of a large pool west of Palm Crossing (see Table 7). That evening, it was found moving through Typha along the northern bank of the pool and was hand-captured for transmitter replacement surgery. The snake was in good condition, and at 2,850g and 256cm total length, had put on 600g and 23cm since initial capture. Its' old transmitter was neatly encapsulated by tissue, exactly as planned, and a new AI-T2 VHF transmitter was fitted. It was released at point of capture on the night of 24/05/2023 and remained unsighted in the northern Typha reedbed over the next two nights (see Table 7

Table 7. Known locations of POP 102.

Date	Location	Latitude	Longitude	Accuracy*	Comments
11/01/2022	Palm Crossing	-21.5694	117.0554	+/- 2m	Found at base of ironstone ridgeline, moving into Typha. Initial capture.
8/06/2022	Palm Crossing	-21.5724	117.0549	+/- 2m	Tracked by Dr. David Pearson (DBCA).
17/06/2022	Palm Crossing	-21.5674	117.0533	+/- 2m	Tracked by Dr. David Pearson (DBCA).
18/06/2022	Palm Crossing	-21.5674	117.0533	+/- 2m	Same location as previous day. Tracked by Dr. David Pearson (DBCA).
22/06/2022	Palm Crossing	-21.5724	117.0549	+/- 2m	Tracked by Dr. David Pearson (DBCA).
12/08/2022	Palm Crossing	-21.5724	117.0549	+/- 2m	Same location as previous – two months apart. Tracked by Dr. David Pearson (DBCA).

Date	Location	Latitude	Longitude	Accuracy*	Comments
13/08/2022	Palm Crossing	-21.5724	117.0549	+/- 2m	Same location as previous day. Tracked by Dr. David Pearson (DBCA).
26/10/2022 1100 hrs	Palm Crossing	-21.5688	117.0529	+/- 2m	In same ironstrone crevice as POP 103. Not observed. Tracked by BHP.
26/10/2022 1930 hrs	Palm Crossing	-21.5688	117.0529	+/- 2m	Same location as morning, still with POP 103. Not observed. Tracked by BHP.
23/05/2023 1100 hrs	Palm Crossing	-21.5710	117.0515	+/- 5m	In Typha reedbed in centre of pool. Position triangulated from two sites on northern bank and one site on southern bank.
23/05/2023 1915 hrs	Palm Crossing	-21.5699	117.0523	+/- 2m	Found active, moving through reedbed on northern bank of pool. Hand captured for transmitter replacement surgery. Appeared healthy.
25/05/2023	Palm Crossing	-21.5701	117.0521	+/- 5m	In Typha on northern edge of pool. Not observed.
26/05/2023	Palm Crossing	-21.5701	117.0521	+/- 5m	In Typha on northern edge of pool. Not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

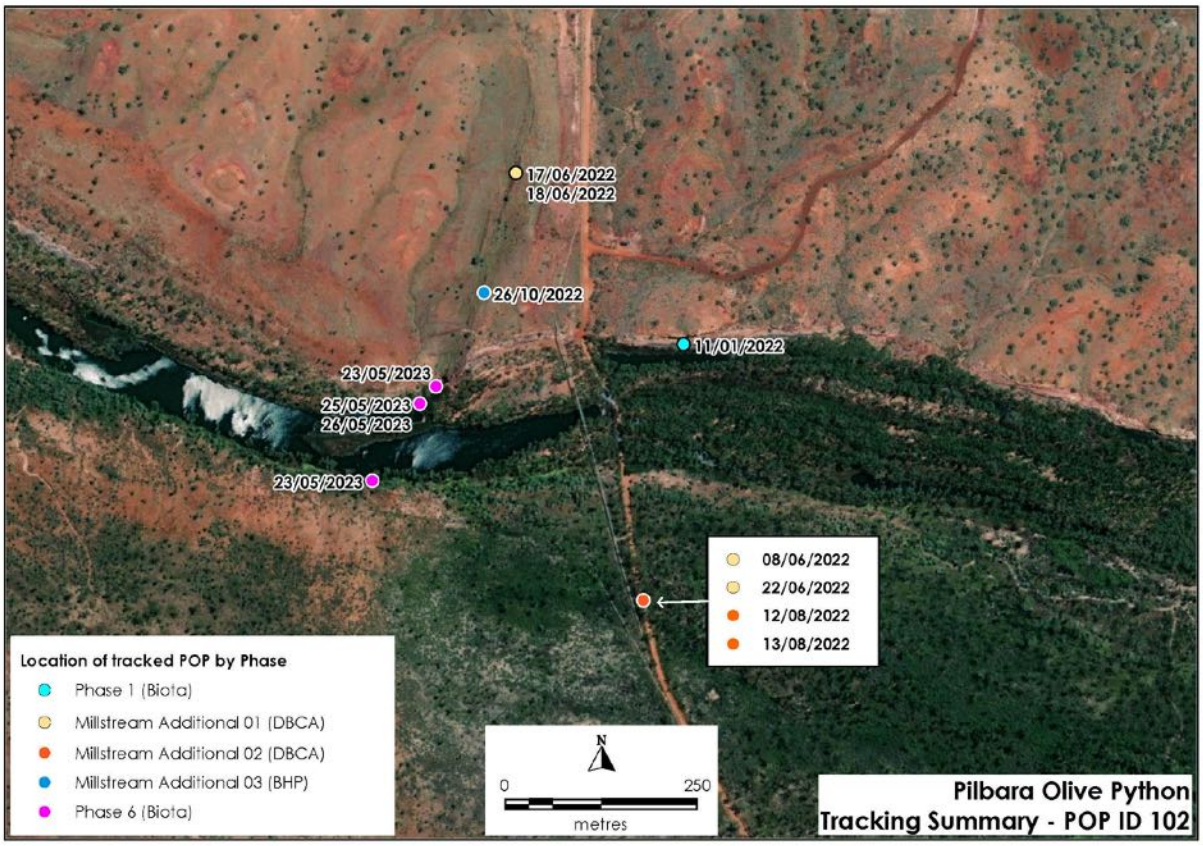

Figure Error! No text of specified style in document..**10. Records of radio-signal tracking locations for POP 102 at Millstream.**

POP 103, a sub-adult male, was first captured on 12/01/22, during phase 1 in a creek west of the track at Palm Crossing. It was fitted with a 12g SI-T2 VHF transmitter and released on 13/01/22 at point of capture and was not re-tracked in Phase 1. No signal was detected for this animal in either June or August 2022 by DBCA's Dr. David Pearson. On 26/10/22, it was tracked by BHP staff Dr. Matt Love, Jared Leigh, Tanya Carroll and Suzi Wild, to an ironstone crevice west of the Palm Crossing track; the same crevice as female POP 102 (see Figure Error**! No text of specified style in document.**.11). Neither were sighted. During phase 6 this animal was detected and located on three consecutive days and nights at Palm Springs deep within a crevice, but the animal was not sighted (see Table 8).

Table 8. Known locations of POP 103.

Date	Location	Latitude	Longitude	Accuracy*	Comments
12/01/2022	Palm Crossing	-21.5700	117.0543	+/- 2m	Found in creek west of Palm Crossing track. Initial capture.

26/10/2022 1100 hrs	Palm Crossing	-21.5688	117.0529	+/- 2m	In same ironstrone crevice as POP 102. Not observed. Tracked by BHP.
26/10/2022 1930 hrs	Palm Crossing	-21.5688	117.0529	+/- 2m	Same location as morning, still with POP 102. Not observed. Tracked by BHP.
23/05/2023 1100hrs	Palm crossing	-21.569591	117.052582	+/- 2m	Tracked to within E facing rocky crevice on ridgeline. Not observed
24/05/2023 1840hrs	Palm crossing	-21.569591	117.052582	+/- 2m	Same location as previous record. Not observed.
25/05/2023 1415hrs	Palm crossing	-21.569591	117.052582	+/- 2m	Same location as previous record. Not observed.
25/05/2023 1900hrs	Palm crossing	-21.569591	117.052582	+/- 2m	Same location as previous record. Not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

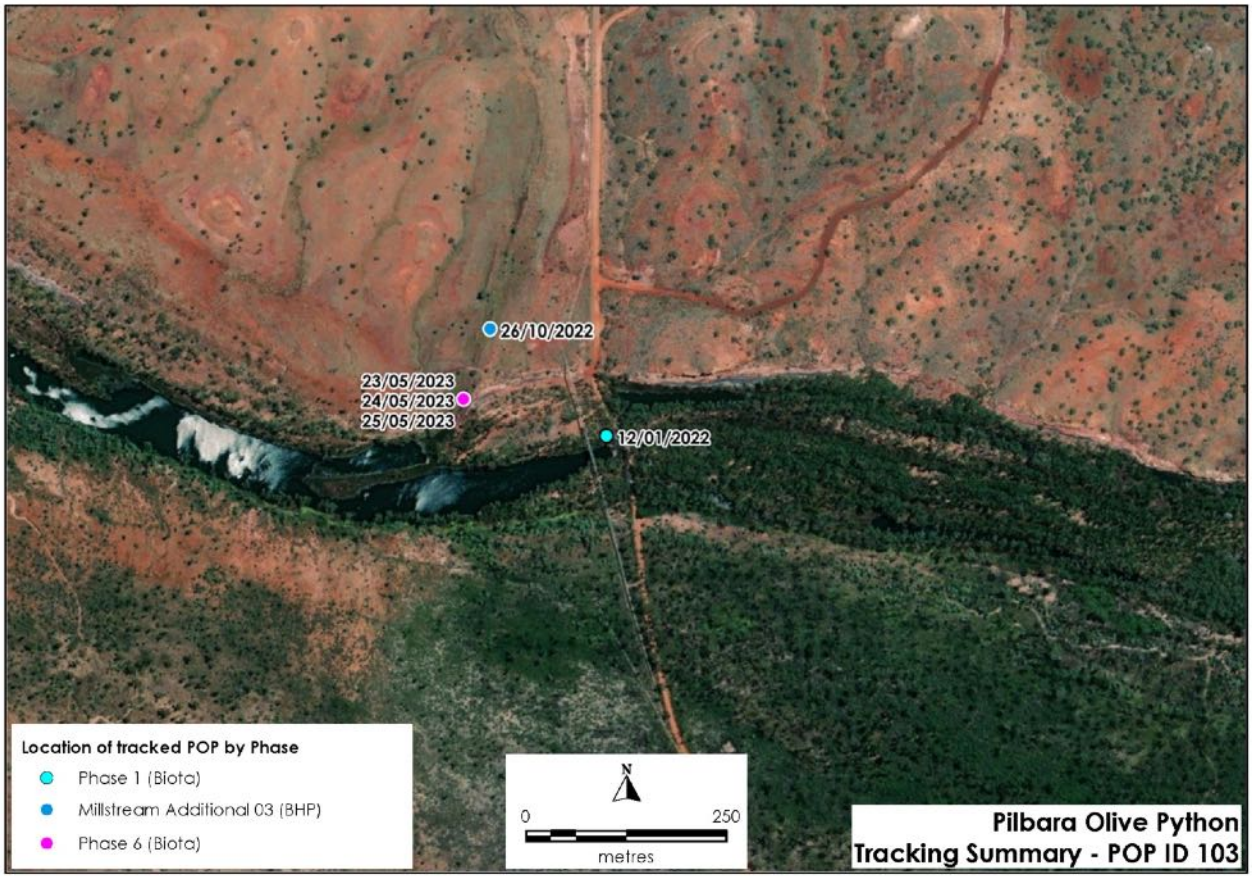

Figure Error! No text of specified style in document..**11. POP 103 radio-tracked locations at Millstream.**

POP 104, a sub-adult female, was first captured on 12/01/22, during phase 1 in a creek west of Miliyanha Campground. It was fitted with a 12g SI-T2 VHF transmitter and released on 13/01/22 at point of capture and was not re-tracked in phase 1. In June 2022, it was tracked by DBCA's Dr. David Pearson to a N-aspect rise (known as "Central rise") ~950m NNW of its previous location. It remained stationary across all three days of tracking; 16, 21 and 22 June 2022. No further signals have been detected for this snake, by either Dr. Pearson in August 2022, BHP in October 2022, or Biota in Phase 6 (May 2023) (see Table 9 and Figure **Error! No text of specified style in document.**.12).

Table 9. Known locations of POP 104.

Date	Location	Latitude	Longitude	Accuracy*	Comments
12/01/2022	Creek west of Miliyanha Campground	-21.5877	117.0707	+/- 2m	Found in creek west of Miliyanha Campground. Initial capture.
16/06/2022	Central rise	-21.5796	117.0680	+/- 2m	N-aspect rise, 950m NNW of its previous location. Tracked by Dr. David Pearson (DBCA).
21/06/2022	Central rise	-21.5796	117.0680	+/- 2m	Same location as previous. Tracked by Dr. David Pearson (DBCA).

22/06/2022	Central rise	-21.5796	117.0680	+/- 2m	Same location as previous. Tracked by Dr. David Pearson (DBCA).

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

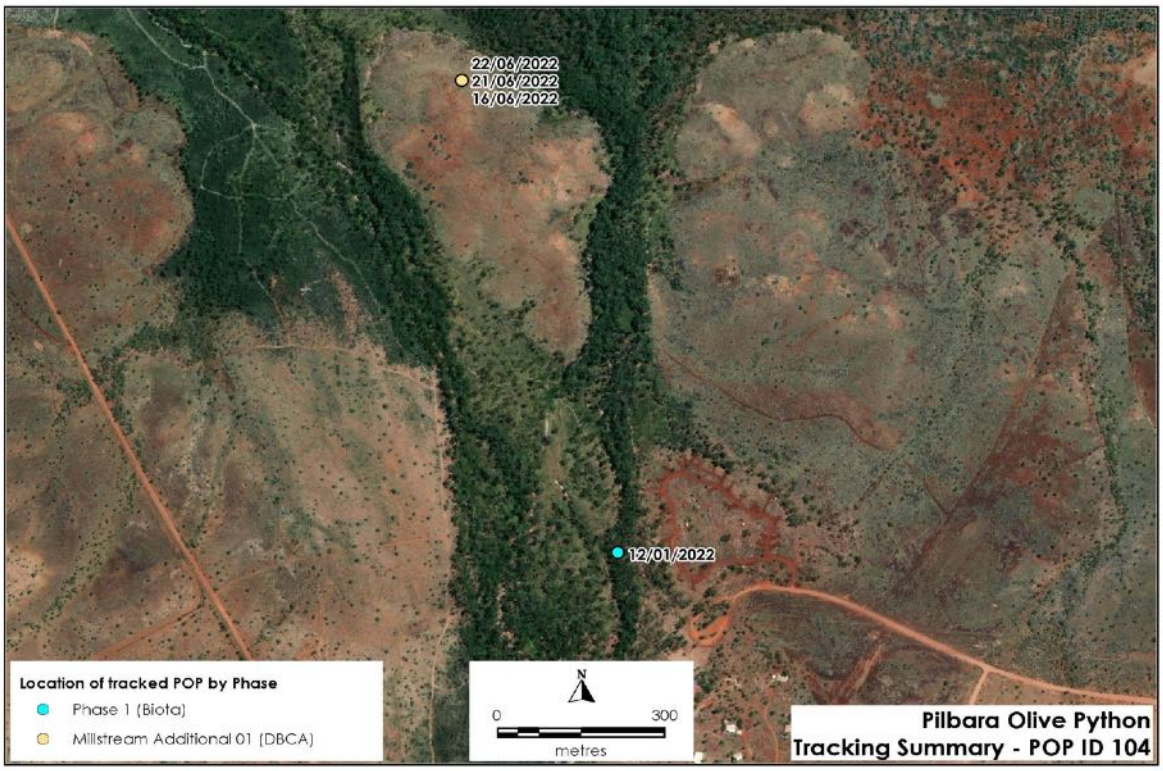

Figure Error! No text of specified style in document..**12. POP 104 radio-tracked locations at Millstream.**

POP 105, a juvenile male, was first captured on 12/01/22, during phase 1 in a on dry ground adjacent to a creek west of Miliyanha Campground. It was fitted with a 10g SI-T2 VHF transmitter and released on 13/01/22 at point of capture and was not re-tracked in phase 1. In June 2022, it was tracked by DBCA's Dr. David Pearson to the roof of the Millstream Homestead, ~350m SE of its previous location, where it remained across all three days of tracking; 13, 16, and 21 June 2022. No signals were detected for this snake by Dr. Pearson in August 2022. In 25 and 26 October 2022, it was tracked by BHP on two days, to a thickly-vegetated creekline 730m NNW of the Millstream Homestead. It was not observed on either occasion. No signal for the snake was detected in phase 6 (May 2023) (see Table 10 and Figure **Error! No text of specified style in document.**.13).

Table 10. Known locations of POP 105.

Date	Location	Latitude	Longitude	Accuracy*	Comments
12/01/2022	Creek west of Miliyanha Campground	-21.5871	117.0708	+/- 2m	Found on dry ground adjacent to a creek west of Miliyanha Campground. Initial capture.
13/06/2022	Roof of Millstream Homestead	-21.5900	117.0720	+/- 2m	In roof of Millstream Homestead, 350m SE of its capture location. Not observed. Tracked by Dr. David Pearson (DBCA).
16/06/2022	Roof of Millstream Homestead	-21.5796	117.0680	+/- 2m	Same location as previous. Tracked by Dr. David Pearson (DBCA).
21/06/2022	Roof of Millstream Homestead	-21.5796	117.0680	+/- 2m	Same location as previous. Tracked by Dr. David Pearson (DBCA).
25/10/2022	Creek NW of Miliyanha Campground	-21.5837	117.0708	+/- 5m	In thickly-vegetated creekline 730m NNW of its previous location. Not observed. Tracked by BHP.
26/10/2022	Creek NW of Miliyanha Campground	-21.5839	117.0706	+/- 5m	Within 20m of the previous location, in same thickly-vegetated creekline. Not observed. Tracked by BHP.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

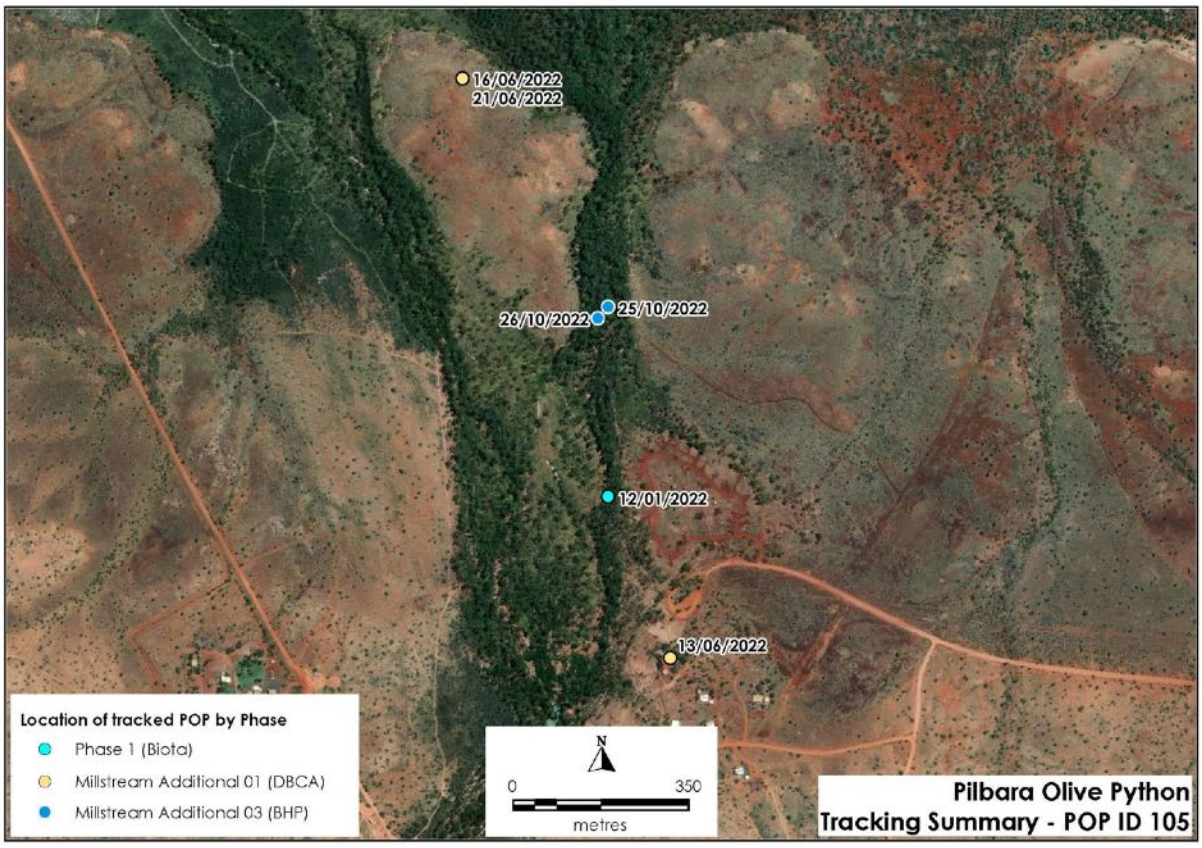

Figure Error! No text of specified style in document..**13. POP 105 radio-tracked locations at Millstream.**

POP 106, a subadult male, was first captured on 13/01/22, during phase 1 beneath a palm tree at the Deep Reach Pool picnic area. It was fitted with a 28g AI-T2 VHF transmitter and released on 14/01/22 at point of capture and was not re-tracked in Phase 1. In June 2022, it was tracked by DBCA's Dr. David Pearson to a location 2km NNE of its previous location, where it remained on the first two days of his tracking; 9 and 21 June 2022. According to the coordinates provided, on 22 June, it had moved 3km east. We would like to follow up further on these coordinates before confirming that the snake move such a distance in 24 hrs. No signals were detected for this snake by Dr. Pearson in August 2022. On 26 October 2022, its signal was detected by BHP staff Matt Love, Jared Leigh, Tanya Carroll and Suzi Wild from the western side Deep Reach Pool. The signal was coming from the eastern side of the pool, and the snake's location has been estimated. In phase 6, on 25 May 2023, it was relocated beneath a conglomerate boulder on the edge of a dry creekbed, 450m south of its location on 9 and 21 June 2022 (see Table 11 and Figure **Error! No text of specified style in document.**.14). It was recaptured for transmitter replacement surgery and was in good condition; at 1,850g and 218cm, it had put on 810g and 38cm since initial capture. Its' original transmitter had been neatly encapsulated by body tissue, exactly as intended. It was fitted with a new AI-T2 VHF transmitter and released at point of capture the following evening.

Table 11. Known locations of POP 106.

Date	Location	Latitude	Longitude	Accuracy*	Comments
12/01/2022	Deep Reach Pool picnic area	-21.6071	117.1059	+/- 2m	Found beneath a palm tree at Deep Reach Pool picnic area. Initial capture.
09/06/2022	NE of Deep Reach Pool	-21.5898	117.1113	+/- 2m	2km NNE of initial capture location. Tracked by Dr. David Pearson (DBCA).
21/06/2022	NE of Deep Reach Pool	-21.5898	117.1113	+/- 2m	Same location as previous. Tracked by Dr. David Pearson (DBCA).
22/06/2022	NE of Deep Reach Pool	-21.5934	117.1394	-	2.9km E of previous location. Tracked by Dr. David Pearson (DBCA).[1]

[1] This is possibly an erroneous record and requires additional follow up with Dr. Dave Pearson.

Date	Location	Latitude	Longitude	Accuracy*	Comments
26/10/2022	East of Deep Reach Pool	-21.6049	117.1072	+/- 200m	Detected at 7-8 bar strength from W side of Deep Reach Pool at (-21.6053, 117.1056). Location provided is an estimate of location and may not be correct. Tracked by BHP.
24/05/2023	NE of Deep Reach Pool	-21.5935	117.1106	+/- 2m	Beneath conglomerate boulder on edge of dry creekbed, 450m S of Dr. Pearson's records on 9 and 21 June 2022. Captured for transmitter replacement surgery and released at this location the following evening.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

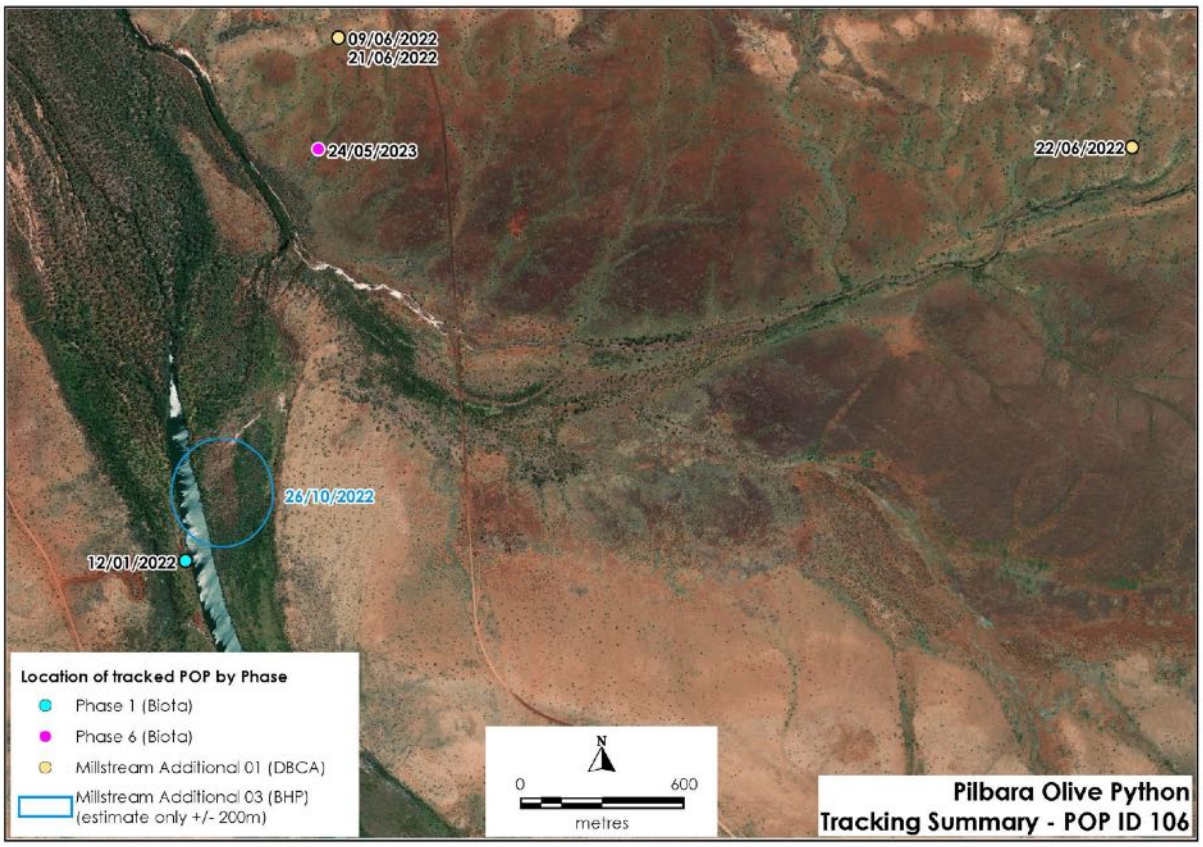

Figure Error! No text of specified style in document..**14. POP 106 radio-tracked locations at Millstream.**

1.3 Ophthalmia Dam

POP 203, an adult female, was first captured on 26/02/22, during phase 2 on the north-eastern edge of the Typha reedbed. Two attempts were made to track her during phase 2, on 28/02/22 and 02/03/22. On both occasions, her signal was detected well, emanating from the offshore Typha. In phase 3, the water level had had greatly receded, allowing for triangulation of signals within the Typha. One attempt was made to track her on 10/12/22, and her signal was successfully triangulated within the reedbed. In phase 4, three attempts were made to track her (see Figure **Error! No text of specified style in document.**.15), one of which was successful; on 25/01/23 she was observed, recaptured, weighed, given a health assessment and released. She had moved into low undulating rocky spinifex habitat, 770m north of the Typha and was found extended amongst the spinifex (see Plate 14 and **Error! Reference source not found.**). It was in excellent condition, and at 3.8kg had put on 1.6kg since her initial capture. It did not appear to be gravid or digesting a recent meal. The microchip scanned well, and surgical wound was almost invisible (see Plate 15). On the following two tracking attempts (28 and 29 January 2023), no signal was detected from the python from either the Typha reedbed, or the area she had previously been located. On the first night of phase 5 (10 May 2023), POP 203 was found to have moved to a crevice on the NNW aspect of an ironstone ridgeline, ~900m NW of the Typha reedbed and 600m W of its last known location (**Error! Reference source not found.**). It was in the same location the following afternoon, and at 20:40 that night, was found 400m S, moving

towards the dam (see **Error! Reference source not found.**, Table 12). It was recaptured for a transmitter replacement surgery, and released the following night (12 May), in the ironstone crevice that it had spent the last two days. At 3.7kg, it was 100g lighter than when re-weighed in January 2023, but was still 1.5kg heavier than its initial capture weight and was in good condition. On both 13 and 14 May, it was found in a NW aspect hole, 40m west of its release point along the same ridgeline (**Error! Reference source not found.**).

Plate 14. **POP 203, as found on 25/01/2023 – in low undulating rocky spinifex habitat.**

Plate 15. **Surgical scar of POP 203, as found on 25/01/2023, 11 months post-surgery. The scar has healed very well and is barely visible.**

Plate 16. **POP 203 as found on 10/05/2023; coiled within NNW aspect of BIF ridgeline.**

Plate 17. **POP 203 as found on 11/05/2023; moving through mallee floodplain towards the dam.**

Table 12. **Known locations of POP 203.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
26/02/2022	Shoreline north-east of Typha reedbed	-23.3519	119.8994	+/- 2m	Found beneath a tree on dry swamp edge. Initial capture.
28/02/2022	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
02/03/2022	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.

Date	Location	Latitude	Longitude	Accuracy*	Comments
10/12/2022	Typha reedbed	-23.3521	119.8983	+/- 15m	Triangulated location within Typha reedbed. Animal not observed.
25/01/2022	Low undulating rocky spinifex habitat	-23.3457	119.9012	+/- 2m	Found in rocky spinifex habitat, recaptured for a health assessment and released immediately.
10/05/2023	Ridgeline NNW of Typha	-23.3452	119.8956	+/- 2m	Found in small crevice on NNW facing aspect of BIF ridgeline, ~900m NW of Typha reedbed. Appeared healthy. 5m from male POP 214, who was in a different crevice.
11/05/2023 1320 hrs	Ridgeline NNW of Typha	-23.3452	119.8956	+/- 2m	In same crevice as yesterday, not observed.
11/05/2023 2040 hrs	Mallee floodplain NW of Typha	-23.3488	119.8961	+/- 2m	Found moving across open mallee floodplain towards dam. Captured for transmitter replacement surgery, and released the following night into the crevice shelter it had used for the past two days.
13/05/2023	Ridgeline NNW of Typha	-23.3452	119.8956	+/- 2m	Found coiled in small NW aspect hole along BIF ridgeline, 40 m W of yesterday's release site. Appeared healthy.
14/05/2023 1600 hrs	Ridgeline NNW of Typha	-23.3452	119.8956	+/- 2m	Sunbaking with head emerged at entrance to same hole as yesterday. Appeared healthy.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

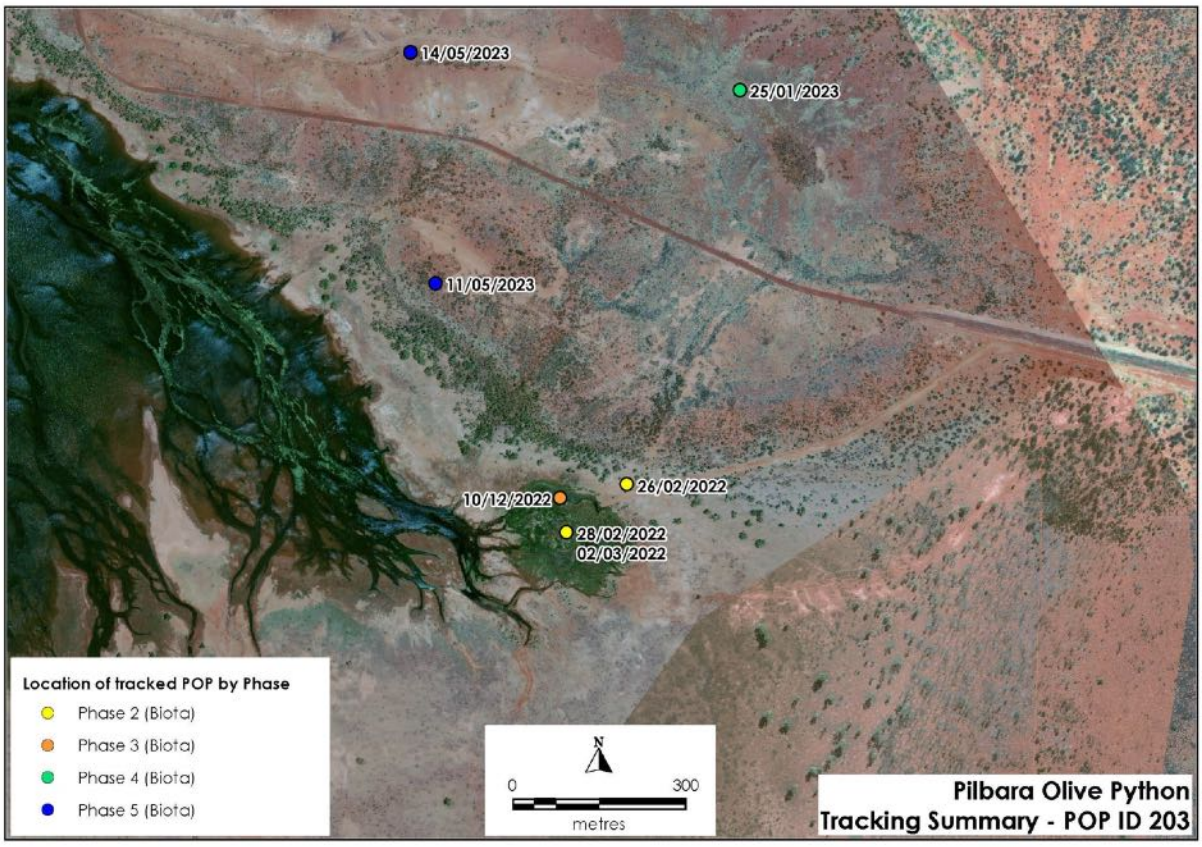

Figure Error! No text of specified style in document.**.15. POP 203 radio-tracked locations at Ophthalmia Dam.**

POP 204, an adult male, was first captured in phase 2 on 26/02/22, near the dams edge, north of the Typha reedbed (see Table 9). It was in a hunting position; submerged in the water, with head just exposed and resting on the edge of a low branch onto which a waterbird might land. Two attempts were made to track him in phase 2, on 28/02/22 and 02/03/22. On both occasions, his signal was detected offshore, in the Typha reedbed (Figure **Error! No text of specified style in document.**.16). Although it was detected in different directions within the reedbed, high water levels prevented accurate triangulation. In phase

3, one attempt, on 10/12/22, was made to track POP 204. Lower water levels allowed for tracking south of the Typha reedbed, which revealed that this animal was further into the dam, south-west of the Typha, likely swimming or submerged. Its location was estimated by triangulation, but only from two points. In phase 4, three attempts were made to track the python; on 25, 28 and 29 January 2023. On each occasion, the animal was not triangulated within the Typha reedbed and not observed. In phase 5 (10-14 May 2023), its' signal could not be detected from the Typha reedbed or surrounding high points. However, on 11 May, its signal was detected SE from a ridgeline near the Ophthalmia Dam creek crossing (-23.3155, 119.8695). The following night, the animal was relocated 3.5 km to the SE of that ridgeline; 1.5 km NW of the Typha reedbed (Plate 18). It was moving along the N edge of an ironstone ridge (Plate 19). Despite an 800g weight loss since its first capture (it now weighed 8000g, vs 8,800g), it appeared to be in very good condition, and surgical wound had healed well. At 4.30pm on May 14, it was relocated 20m west of its last location, in a N-facing crevice on the same ridgeline.

Plate 18. **POP 204, as found on 12/05/2023 – at northern base of a BIF ridgeline.**

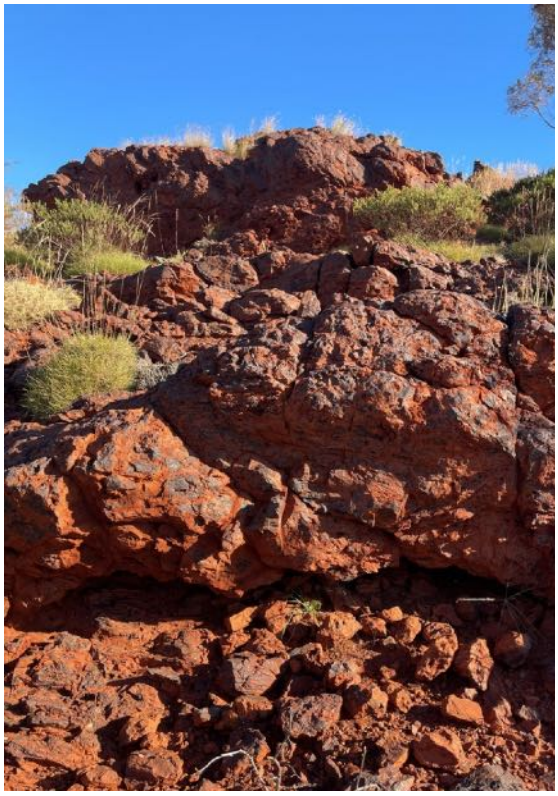

Plate 19. **N-aspect crevice which POP 203, was found in on 14/05/2023.**

Table 13. **Known locations of POP 204.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
26/02/2022	In dam, north of Typha reedbed	-23.3514	119.8980	+/- 2m	Found almost fully submerged in a hunting position, with head resting on a low branch which a waterbird might land on. Initial capture.
28/02/2022	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
02/03/2022	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
10/12/2022	In dam, south-west of Typha reedbed	-23.3533	119.8975	+/- 100m	Offshore in dam, south-west of Typha reedbed. Location estimated from two triangulation points.
25/01/2023	Typha reedbed	-23.3524	119.8976	+/- 15m	Triangulated within Typha reedbed
28/01/2023	Typha reedbed	-23.3527	119.8977	+/- 10m	Triangulated within Typha reedbed
29/01/2023	Typha reedbed	-23.3524	119.8977	+/- 15m	Triangulated within Typha reedbed

Date	Location	Latitude	Longitude	Accuracy*	Comments
12/05/2023	Ridgeline NW of Typha	-23.3419	119.8896	+/- 2m	Found moving along open ground on north side of a BIF ridgeline, 1.5km NW of Typha reedbed. Recaptured and kept overnight for health assessment, before being released the following night at point of capture.
14/05/2023 1630 hrs	Ridgeline NW of Typha	-23.3418	119.8894	+/- 2m	In N-aspect rocky crevice, 20 m from release point. Not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

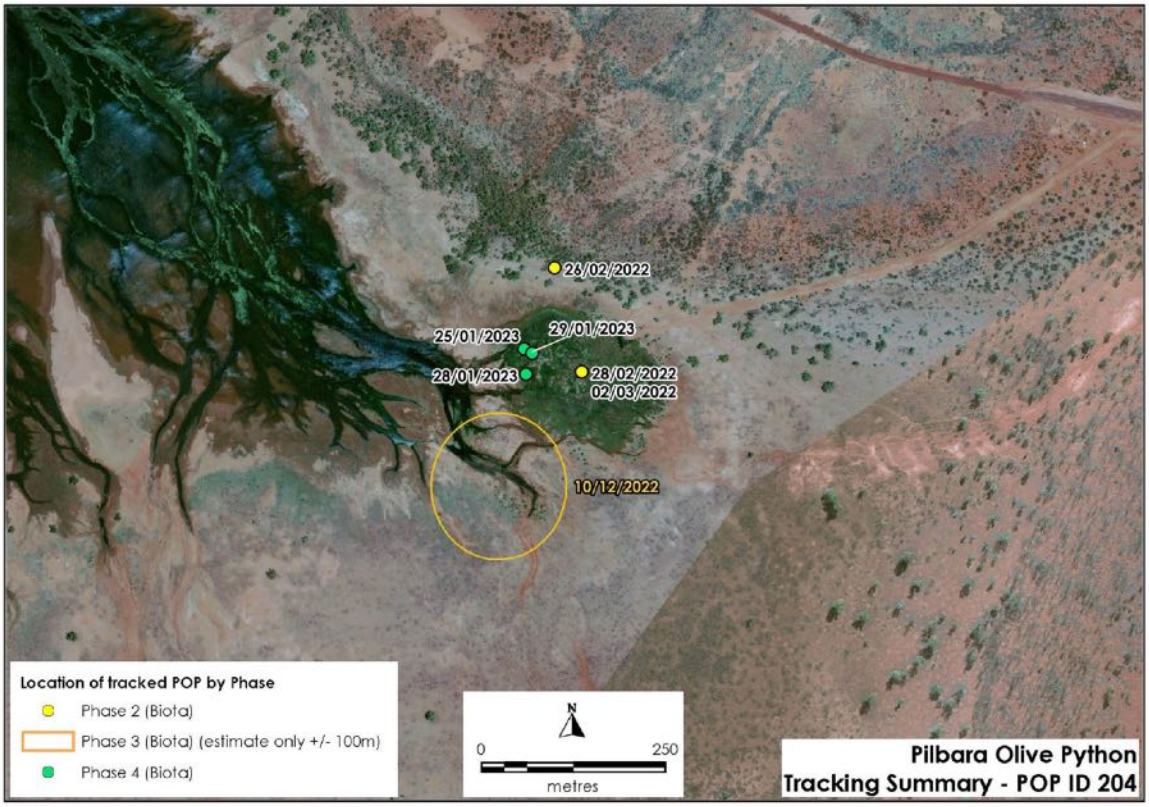

Figure Error! No text of specified style in document..**16. Records of radio-signal tracking locations for POP 204 at Ophthalmia Dam.**

POP 205, a juvenile male, was first captured in phase 2 on 27/02/22, north of the creek crossing on the Ophthalmia Dam entry track. He was found on the edge of a shallow pool, alongside a larger creek which contained water along its full extent. Following release on 28/02/22, one attempt was made to track him in phase 2, on 02/03/22. In the 48 hours between release and relocation, he had moved ~750m northwards, likely following the creekline, moving north of the Jimblebar rail-line, which we did not have approval to cross. Its' location was estimated from south of the Jimblebar rail-line, based on direction and signal strength. In phase 3, on 10 December 2022, it was found deceased near its original capture point (Figure **Error! No text of specified style in document.**.17, Table 14). However, on this survey, the creek and all associated pools were completely dry. The body was desiccated, covered in ants, and exposed on a raised island between two now-dry pools (see Plate 20). The microchip was still scannable within the body, and VHF transmitter was located around a metre away, in fine condition. It is likely that the harsher conditions caused by the dried habitat contributed to this young individual's death.

Plate 20. **The deceased body of POP 205, as found on 10/12/2022 – exposed on a raised island between two now-dry pools.**

Table 14. **Known locations of POP 205.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
27/02/2022	Creek north of road crossing	-23.3118	119.8674	+/- 2m	Found on the edge of a shallow pool, alongside a larger creek. Initial capture.
02/03/2022	North of Jimblebar rail-line	-23.3062	119.8656	+/- 100m	North of Jimblebar rail-line, which we did not have permission to cross. Location estimated based on direction and signal strength. Likely in, or adjacent to, the same creek it was captured in.
10/12/2022	Creek north of road crossing	-23.3111	119.8676	+/- 2m	Found deceased, exposed on a raised island between two now-dry pools.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

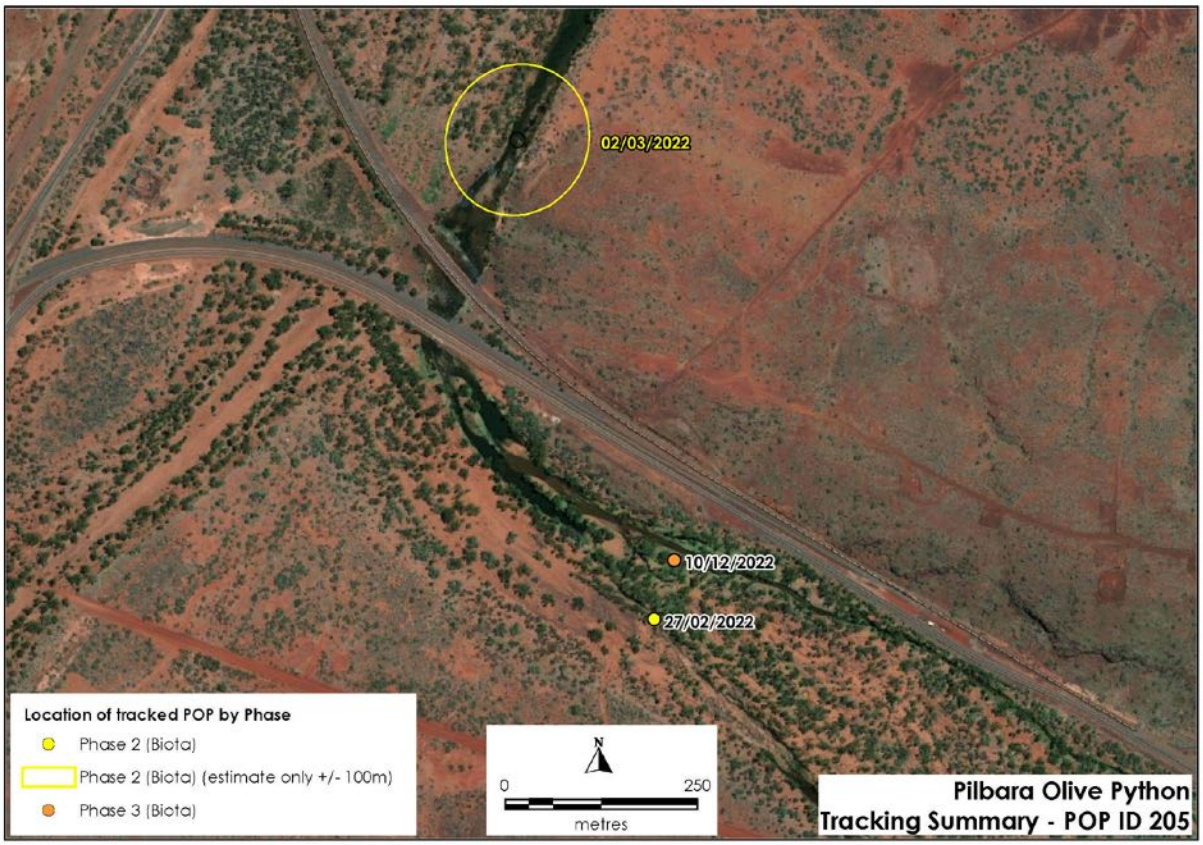

Figure Error! No text of specified style in document..**17. Capture location of POP 205 (Feb 2022), radiotracking attempts and locality of deceased individual (Dec 2022).**

POP 206, an adult male, was first captured in phase 2 on 28/02/22, resting on dry ground beneath a tree, north of the Typha reedbed (

Table 15). The first attempt to track this snake occurred in phase 3, on 10 December 2022, and it was triangulated within the Typha reedbed. In phase 4, three attempts were made to track the python; on 25, 28 and 29 January 2023 (see Figure **Error! No text of specified style in document.**.18). On January 25, it was triangulated within the Typha reedbed. On January 28, it was found in an ambush position south-west of the Typha reedbed; in a dead tree with head resting at the base of flat branch, ready to hunt any birds that may land there (Plate 21). The python was captured, weighed, given a health assessment, and then released back into the tree. It weighed 5,045g, a decrease of 2,355g since its initial capture. Despite this weight loss, the python appeared to be healthy and was moving normally. The microchip scanned properly, and surgical scar had healed to the point of being almost invisible (Plate 22). The following day, January 29, its position was triangulated near the south-western edge of the Typha reedbed. By phase 5 (May 2023), the python had moved north-west to a series of low ironstone ridges. On 10 May it was found slithering into a N-facing cavern in a low ridgeline, ~900 m NW of the Typha reedbed (Plate 24). It appeared large, glossy and healthy (Plate 23). From 11 – 14 May, it remained stationary within the NW-facing aspect of an ironstone rockpile, 115 m west of its previous location (Plate 25).

Plate 21. **POP 206 as found on 28/01/2023; in ambush position up a dead tree, head resting at the base of a flat branch, likely waiting for a bird to land.**

Plate 22. **Surgical scar of POP 206, as found on 28/01/2023, 11 months post-surgery. The scar has healed very well and is barely visible.**

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Plate 23. **POP 206, within an ironstone crevice on 10/05/2023.**

Plate 24. **POP 206, as found on 10/05/2023, moving into an ironstone crevice.**

Plate 25. **The rockpile which POP 206 remained in from 11-14/05/2023. It stayed non-visible beneath rocks on the NW aspect.**

Table 15. **Known locations of POP 206.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/02/2022	Shoreline north of Typha reedbed	-23.3521	119.9006	+/- 2m	Found beneath a tree on dry swamp edge. Initial capture.
10/12/2022	Typha reedbed	-23.3522	119.8986	+/- 15m	Triangulated within Typha reedbed
25/01/2023	Typha reedbed	-23.3525	119.8974	+/- 15m	Triangulated within Typha reedbed
28/01/2023	South-west of Typha reedbed	-23.3525	119.8872	+/- 2m	Found in ambush position up a dead tree, with head resting at the base of a flat branch, ready to strike any birds that may land there.
29/01/2023	Typha reedbed	-23.3527	119.8977	+/- 10m	Triangulated within Typha reedbed
10/05/2023	Ridgeline NW of Typha	-23.3459	119.8936	+/- 2m	Found slithering into a N-aspect cavern on a low ironstone ridge, 900 m NW of the Typha reedbed. Appeared large, glossy and healthy.
11/05/2023 13:40 hrs	Rockpile NW of Typha	-23.3457	119.8925	+/- 2m	Located within the NW side of a rockpile, 115 m W of its previous location. Not observed.
11/05/2023 21:10 hrs	Rockpile NW of Typha	-23.3457	119.8925	+/- 2m	In same location as previous, not observed.
12/05/2023	Rockpile NW of Typha	-23.3457	119.8925	+/- 2m	In same location as previous, not observed.
13/05/2023	Rockpile NW of Typha	-23.3457	119.8925	+/- 2m	In same location as previous, not observed.
14/05/2023	Rockpile NW of Typha	-23.3457	119.8925	+/- 2m	In same location as previous, not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

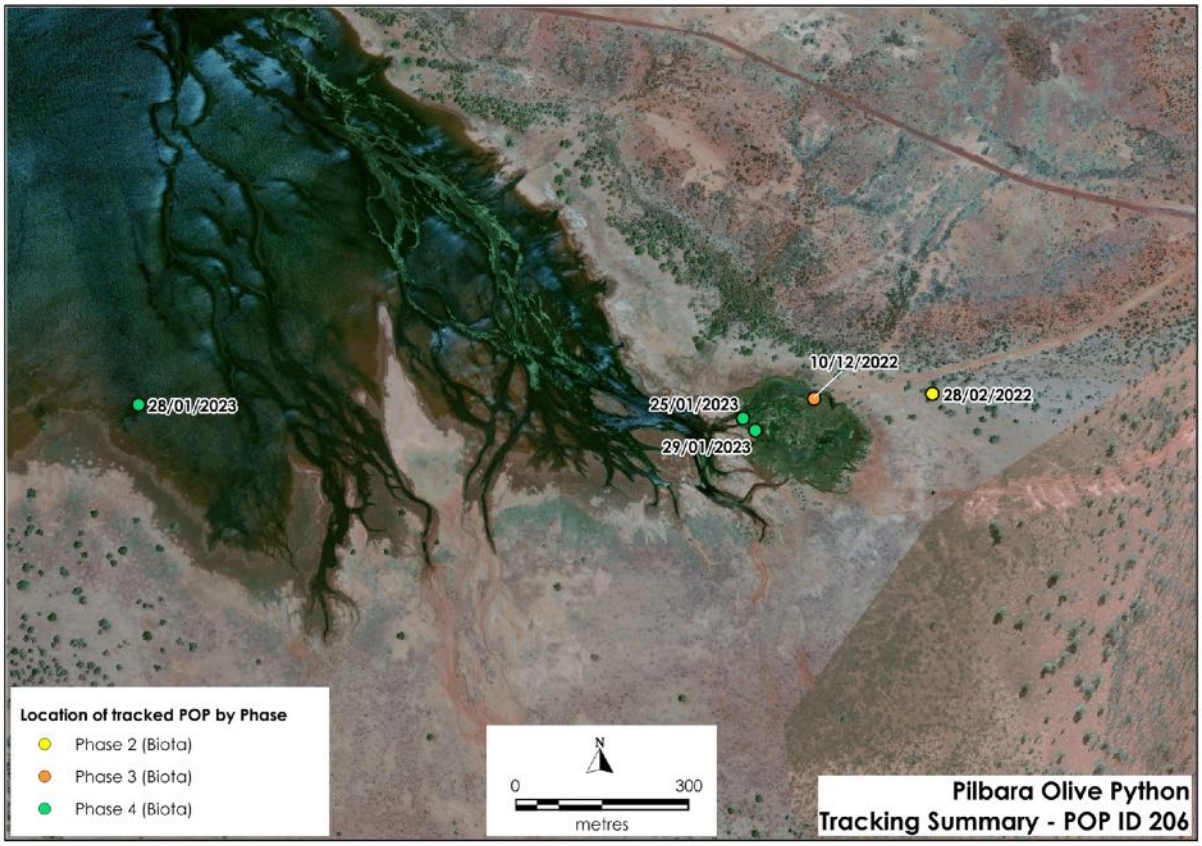

Figure Error! No text of specified style in document..**18. POP 206 radio-tracked locations at Ophthalmia Dam.**

POP 207, an adult female, was first captured in phase 2 on 28/02/22, resting on dry ground south-east of the Typha reedbed (see Table 16, Figure **Error! No text of specified style in document.**.19). The first attempt to track this snake occurred in phase 3, on 10 December 2022. However, no signals were detected either from the Typha reedbed area, or the creek crossing. On the first night of the phase 4 survey, it was resighted and captured without radiotracking: swimming upstream in a narrow stream flowing along the western edge of the Typha reedbed. It was captured and kept for weighing and a health assessment (Plate 26). At 4,555g it was 855g lighter than when first captured 11 months prior and visibly thinner, with no recent meal being digested. The microchip scanned properly, and VHF transmitter was working fine. The surgical scar had healed to the point of being almost invisible (Plate 27). On January 25 it was released back at its point of capture. Two subsequent attempts were made to radio-track the python during phase 4. On January 28, it was relocated in a 2m wide sliver of dense Typha, at the northern edge of the reedbed. Despite our proximity, it could not be observed. On January 29, it was triangulated slightly further south into the reedbed. Throughout phase 5 (10-14 May 2023), it remained stationary beneath rocks on the NW aspect of an outcrop 450m NE of the Typha reedbed (Plate 28, Plate 29). The animal was sloughing, and fresh shed skin found outside its hole on 14 May.

Plate 26. **POP 207 after being recaptured on 23/01/2023.**

Plate 27. **Surgical scar of POP 207, as found on 23/01/2023, 11 months post-surgery. The scar is visible but has healed very well.**

Plate 28. **POP 207 as found on 10/05/2023, sloughing beneath a NW aspect outcrop.**

Plate 29. **The NW aspect outcrop which POP 207 remained in from 10-14 May 2023. It was beneath the large rock on centre-right.**

Table 16. **Known locations of POP 207.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/02/2022	Shoreline south-east of Typha reedbed	-23.3538	119.9038	+/- 2m	Found resting on dry swamp edge. Initial capture.
23/01/2023	Stream west of Typha reedbed	-23.3523	119.8974	+/- 2m	Found swimming upstream in a narrow flowing passage west of the Typha reedbed.
28/01/2023	Typha reedbed	-23.3523	119.8989	+/- 3m	Tracked to a dense 2m wide edge of the Typha reedbed and could not be observed within it.
29/01/2023	Typha reedbed	-23.3525	119.8989	+/- 15m	Triangulated within Typha reedbed
10/05/2023	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Found coiled and sloughing within NW facing rocks atop an outcrop ~450 m NE of Typha reedbed. Appeared healthy, but slough not shedding cleanly off face.
11/05/2023 13:00 hrs	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Seen in same location as previous.

11/05/2023 21:30 hrs	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Seen in same location as previous.
12/05/2023	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Seen in same location as previous.
13/05/2023	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Seen in same location as previous.
14/05/2023	Outcrop N of Typha reedbed	-23.3496	119.9017	+/- 2m	Seen in same location as previous. New shed-skin just outside its rocks indicate that it had moved out and back between observations.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

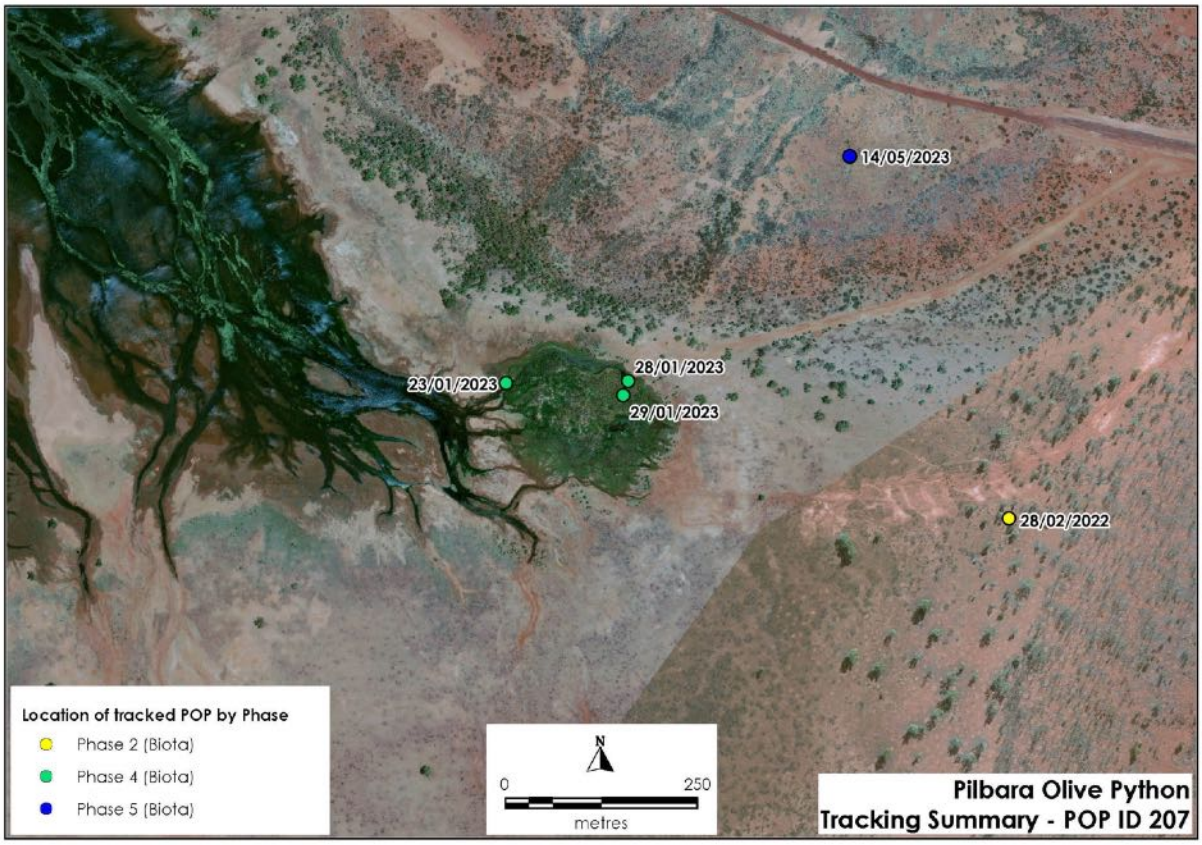

Figure Error! No text of specified style in document..**19. POP 207 capture location, attempted radio-tracking locations and capture locations at Ophthalmia Dam.**

POP 208, an adult male, was first captured in phase 2 on 28/02/22, north of the creek crossing on the Ophthalmia Dam entry track (Table 17). It was found resting, submerged in a pooled creek digesting a meal; assessed by feel as likely being a young macropod. This creek was full and flowing in phase 2, but dry in phases 3 and 4. The first attempt to track this snake occurred in phase 3, on 10 December 2022, but no signals were detected either from the Typha reedbed area, or the creek crossing. During the phase 4 survey, it was successfully tracked three times from three attempts, and sighted each time (Figure **Error! No text of specified style in document.**.20). On 25 January 2023, it was tracked, to the base of an ironstone ridgeline where it was found basking 2m from a deep crevice, 430m south-east of its original capture point (Plate 30). It was recaptured, weighed, given a health assessment, and released. Upon release, it retreated into the deep crevice (see Plate 32). At 3,430g, it was 840g lighter than when first captured, however, this time it was not carrying a large meal. It was recaptured in healthy, glossy condition, with microchip scanning properly and surgery scar visible but well-healed (see Plate 31). On 28 January, it was relocated atop the same ridgeline, 325m west-north-west of its previous location. The animal was found stationary but was likely moving before we interrupted it. On 29 January, it was found basking outside the same deep crevice as on January 25, which is likely a regular shelter (Plate 33 and **Error! Reference source not found.**). The python was covered in water droplets, indicating that it had basked through the rain which fell in the preceding half hour (Plate 34). As it had been

given a health assessment on January 25, it was not handled on January 28 or 29. No signal was detected during phase 5 (May 2023).

Plate 30. **POP 208, as found on 25/01/2023; basking outside a deep crevice.**

Plate 31 **Surgical scar of POP 208, as found on 25/01/2023, 11 months post surgery. The scar is visible but has healed very well (upper left of image). The scar at bottom of image is not associated with surgery.**

Plate 32. **POP 208 retreating into a deep crevice post-release on 25/01/2023. It quickly moved out of sight.**

Plate 33. **POP 208 as found on 28/01/2023, atop an ironstone ridgeline.**

Plate 34. **POP 208 as found on 29/01/2023, basking outside the same crevice (not visible) as on 25/01/2023.**

Table 17. **Known locations of POP 208.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/02/2022	Creek north of road crossing	-23.3123	119.8679	+/- 2m	Found resting, submerged in creek and digesting a meal. Initial capture.
25/01/2023	Ridgeline south of entrance road	-23.3158	119.870	+/- 2m	Found basking outside a deep crevice on the edge of an ironstone ridge.
28/01/2023	Ridgeline south of entrance road	-23.3148	119.8671	+/- 2m	Found stationery atop ironstone ridgeline. Was likely moving before we interrupted it.
29/01/2023	Ridgeline south of entrance road	-23.3159	119.8701	+/- 2m	Found basking outside the same crevice as on 25/01/2023.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

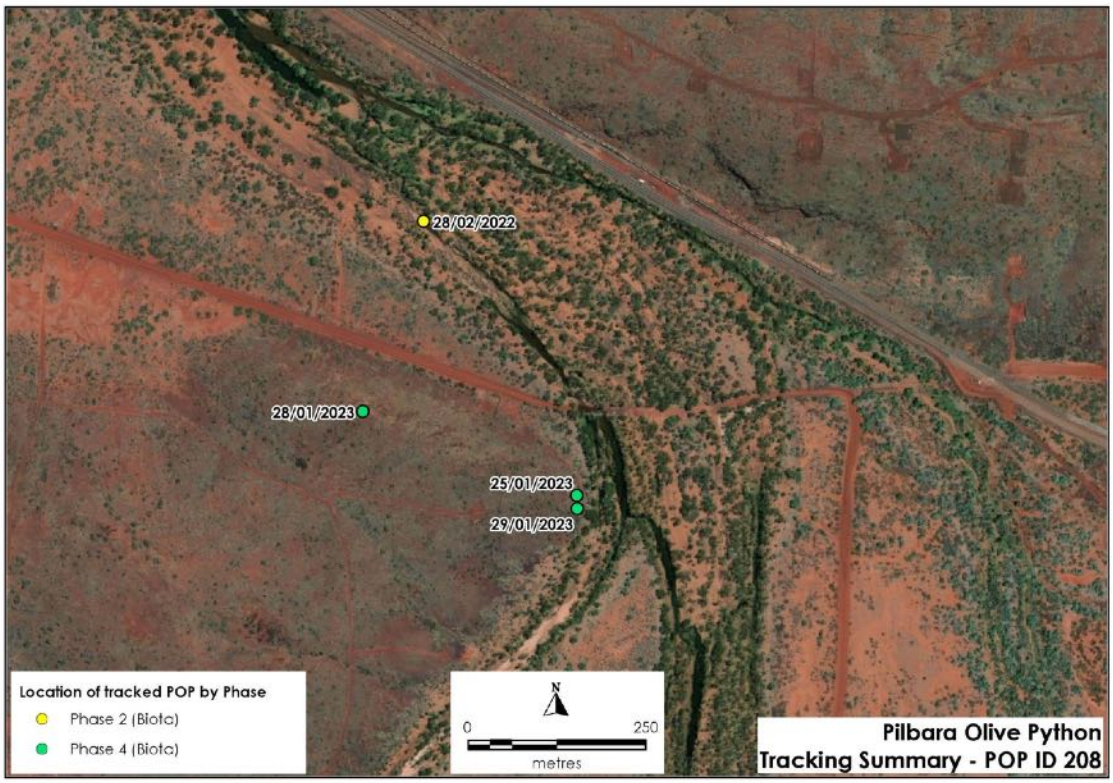

Figure Error! No text of specified style in document..**20. POP 208 radio-tracked locations at Ophthalmia Dam.**

POP 209, a juvenile male, was first captured in phase 2 on 28/02/22, resting on dry ground north of the Typha reedbed (Table 18). The first attempt to track this snake occurred in phase 3, on 10 December 2022, and it was triangulated within the Typha reedbed (Figure **Error! No text of specified style in document.**.21). In Phase 4, three attempts were made to track the python: on 25, 28 and 29 January 2023 (Figure **Error! No text of specified style in document.**.21). On all occasions, it was triangulated within the Typha reedbed, moving from west to east between January 25 and 28, and then remaining near the same location on January 29. No signal was detected during phase 5 (May 2023).

Table 18. Known locations of POP 209.

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/02/2022	Shoreline north of Typha reedbed	-23.3518	119.8994	+/- 2m	Found beneath a tree on dry swamp edge. Initial capture.
10/12/2022	Typha reedbed	-23.3523	119.8987	+/- 15m	Triangulated within Typha reedbed
25/01/2023	Typha reedbed	-23.3526	119.8979	+/- 15m	Triangulated within Typha reedbed
28/01/2023	South-west of Typha reedbed	-23.3527	119.8989	+/- 15m	Triangulated within Typha reedbed
29/01/2023	Typha reedbed	-23.3527	119.8989	+/- 15m	Triangulated within Typha reedbed

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

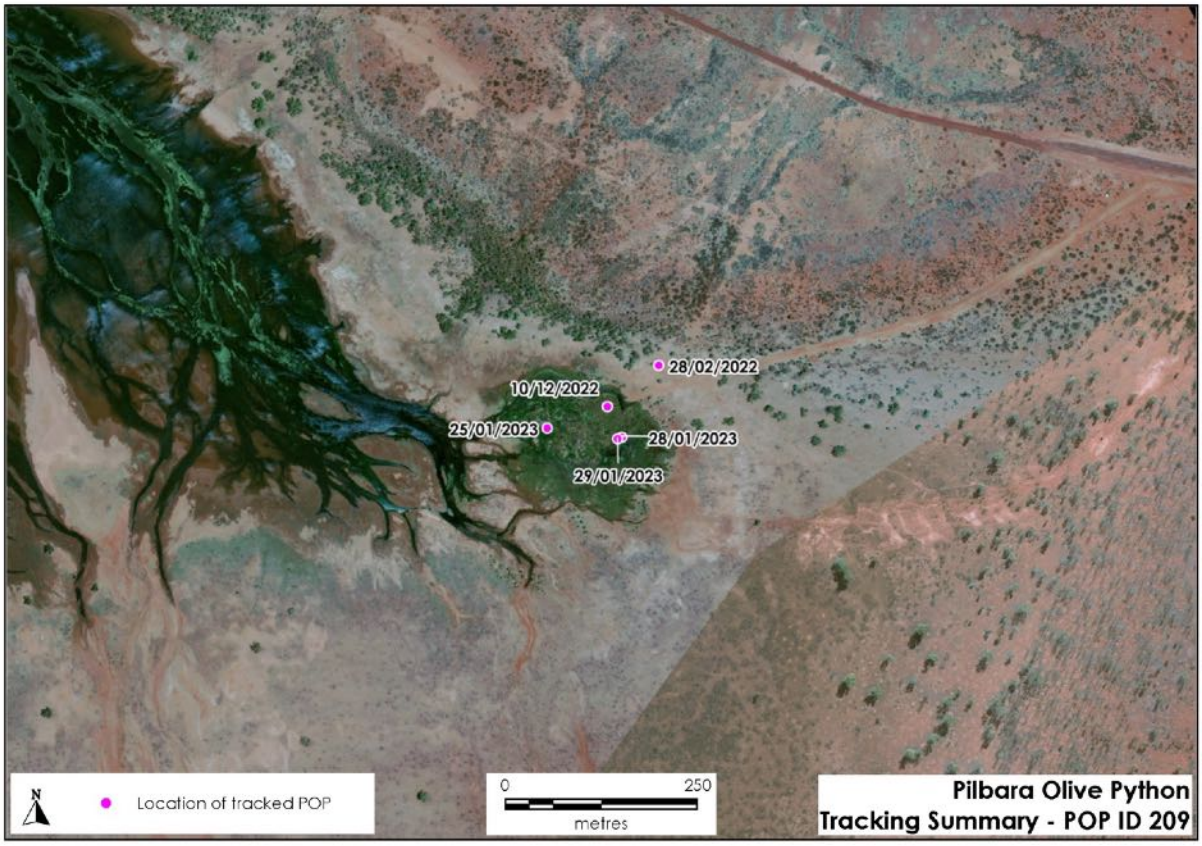

Figure Error! No text of specified style in document..**21. Records of radio-signal tracking locations for POP 209 at Ophthalmia Dam.**

POP 210, a juvenile female, was first captured in phase 2 on 28/02/22, resting on dry ground north of the Typha reedbed (Table 19). The first attempt to track this snake occurred in phase 3, on 10 December 2022, and its position was triangulated within the Typha. During the phase 4 survey, it was successfully tracked on three days, from three attempts (Figure **Error! No text of specified style in document.**.22). On January 25, it was found in an ambush position; hanging from a low branch, camouflaged amongst sticks and debris, with head just above the ground, apparently waiting for a mouse to run past (Plate 35 and Plate 36). House mice (*Mus musculus*) are visibly abundant on the swampy margins of the Typha reedbed. It was captured, weighed, given a health assessment, and released back onto the branches it was caught on. At 530g (excluding its 10g transmitter), it has lost 40g since its initial capture. Whilst thin, it appeared to be in good condition, fine for a young snake. Its microchip scanned properly, and its surgical wound has healed well (Plate 37). On January 28, its signal was tracked to the northern edge of the Typha reedbed, very close to the edge, but the animal could not be seen. On January 29, it was once again hidden right on the edge of the reedbed, this time in the south-west. No signal was detected during phase 5 (May 2023).

Plate 35. **POP 210 as found on 25/01/2023 in an ambush position; hanging from low branch with head poised just above the ground waiting for a mouse to move past (centre of image).**

Plate 36. **POP 210 as found on 25/01/2023 in an ambush position – closer image.**

Plate 37. **Surgical scar of POP 210, as found on 25/01/2023, 11 months post-surgery. The scar is visible but has healed very well.**

Table 19. **Known locations of POP 210.**

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/02/2022	Shoreline north of Typha reedbed	-23.3520	119.9005	+/- 2m	Found resting on dry swamp edge. Initial capture.
10/12/2022	Typha reedbed	-23.3523	119.8988	+/- 15m	Triangulated within Typha reedbed

25/01/2023	Shoreline north of Typha reedbed	-23.3515	119.898	+/- 2m	Found in an ambush position; hanging from a low branch, camouflaged amongst sticks and debris, with head just above the ground, apparently waiting for a mouse to run past.
28/01/2023	Typha reedbed	-23.3524	119.8991	+/- 5m	Tracked to the northern edge of the Typha reedbed.
29/01/2023	Typha reedbed	-23.3527	119.8977	+/- 5m	Tracked to the south-western edge of the Typha reedbed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

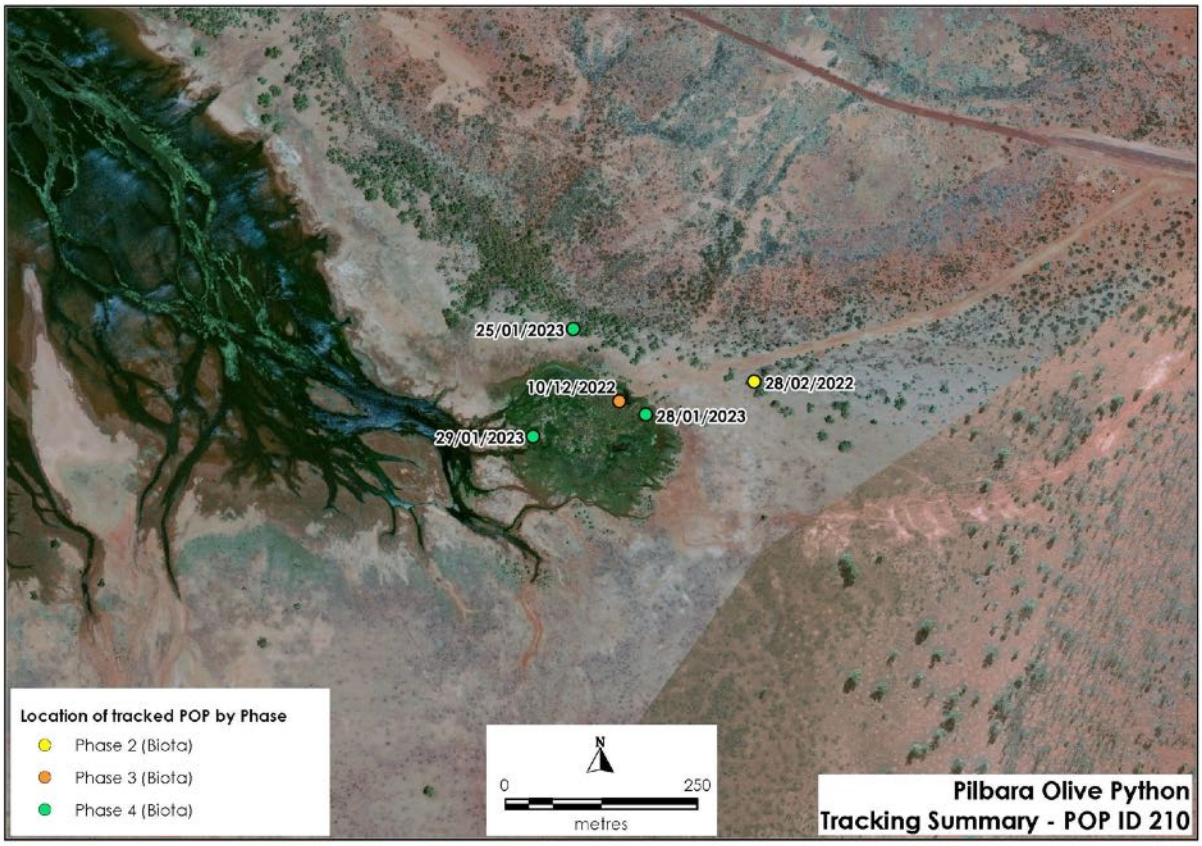

Figure Error! No text of specified style in document..**22. POP 210 radio-tracking locations at Ophthalmia Dam.**

POP 213, an unsexed juvenile, was first captured in phase 3 on 10/12/2022, resting on dry ground south of the Typha reedbed (see Table 20 and Figure **Error! No text of specified style in document.**.23). It was very thin and scale-clipped only, with an estimated weight of 500g. It has no VHF transmitter and has not been resighted.

Table 20. Known locations of POP 213.

Date	Location	Latitude	Longitude	Accuracy*	Comments
10/12/2022	South of Typha reedbed	-23.3535	119.8983	+/- 2m	Found on dry swamp edge. Initial capture.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

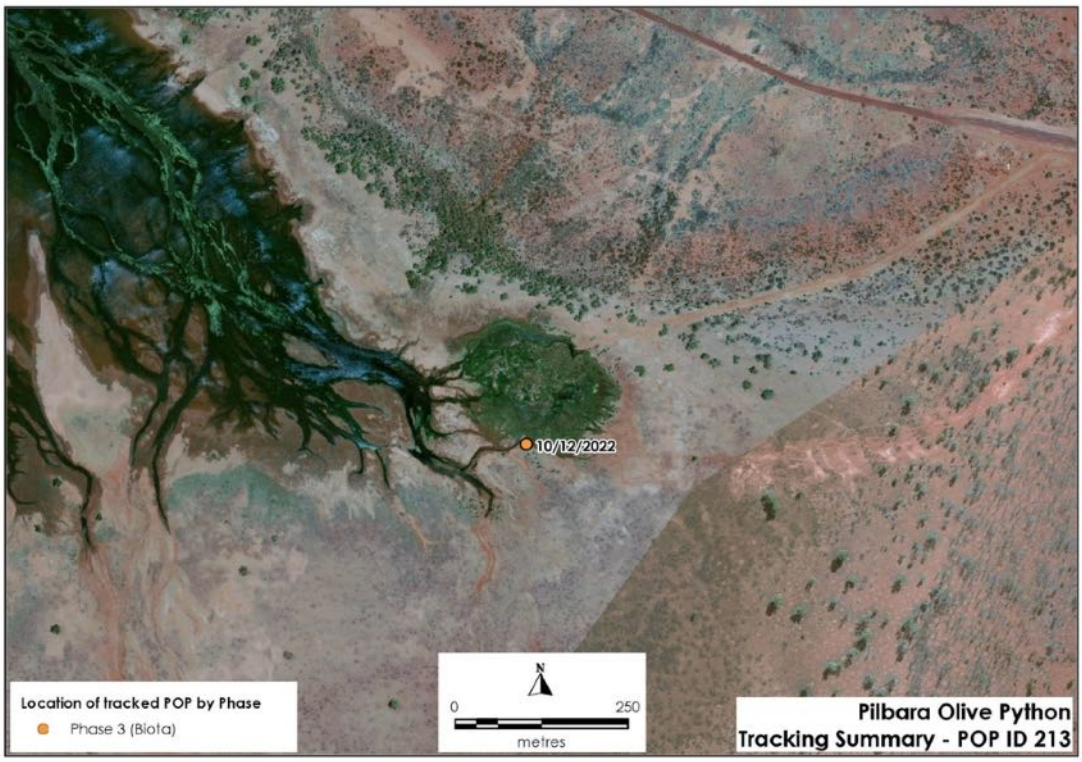

Figure Error! No text of specified style in document..**23. Capture locations of POP 213 at Ophthalmia Dam.**

POP 214, an adult male, was first captured in phase 3 on 10/12/2022, in a dead tree south-west of the Typha reedbed (Table 21). It was initially microchipped and scale-clipped, then released without a VHF transmitter. In phase 4, it was recaptured on the ground 15m from its' initial capture point (see Figure **Error! No text of specified style in document.**.24) fitted with a VHF transmitter and re-released on January 29. It was first tracked in Phase 5 (10-14 May 2023), and first relocated coiled within a crevice on the N-facing aspect of a BIF ridgeline (Plate 38 and Plate 39), 5m from female POP 203. In the afternoon of 11 May it remained in the same location and had moved 40 m W along the same ridgeline by 8:40pm that night. The following night (12 May), it was relocated on a different ridgeline, 130m N (Plate 40). On 13 and 14 May, it remained ~20m from its 12 May location, within a small crevice atop the ridgeline, where large urates (likely from snakes) were present (Plate 41).

Plate 38. POP 214, as found on 10/05/2023, coiled beneath the N aspect of a BIF ridgeline.

Plate 39. Rock which POP 214 was found beneath on the night of 11/05/2023.

Plate 40. **Crevice which POP 214 was located in on 12/05/2023.**

Plate 41. **Crevice which POP 214 was located in on 13 and 14/05/2023.**

Table 21. Known locations of POP 214.

Date	Location	Latitude	Longitude	Accuracy*	Comments
10/12/2022	South-west of Typha reedbed	-23.3530	119.8977	+/- 2m	Found in dead tree, south-west of Typha reedbed. Initial capture.
28/01/2023	South-west of Typha reedbed	-23.3529	119.8976	+/- 2m	Found on dry swamp edge, 15m from previous location. Fitted with VHF transmitter.
10/05/2023	Ridgeline NNW of Typha	-23.3452	119.8957	+/- 2m	Found curled within a crevice on the N-facing aspect of a BIF ridgeline, ~900m NW of Typha reedbed, and 5m from female POP 203 (who was in a separate crevice). Appeared healthy.
11/05/2023 1325 hrs	Ridgeline NNW of Typha	-23.3452	119.8957	+/- 2m	In same location as previous. Not observed.
11/05/2023 2020 hrs	Ridgeline NNW of Typha	-23.3452	119.8953	+/- 2m	Found coiled beneath a rock, 40 m W, on the northern base of the ridgeline it was previously found on. Appeared healthy.
12/05/2023	Ridgeline NNW of Typha	-23.3440	119.8956	+/- 5m	In crevice on the S-aspect of a BIF ridgeline, 130 m N of the ridgeline it was previously on. Not observed.
13/05/2023	Ridgeline NNW of Typha	-23.3439	119.8958	+/- 5m	In crevice atop BIF ridgeline, 20 m from last record. Small crevice with large urates present, likely from snakes. Not observed.
14/05/2023	Ridgeline NNW of Typha	-23.3439	119.8958	+/- 5m	In same location as previous, not observed.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

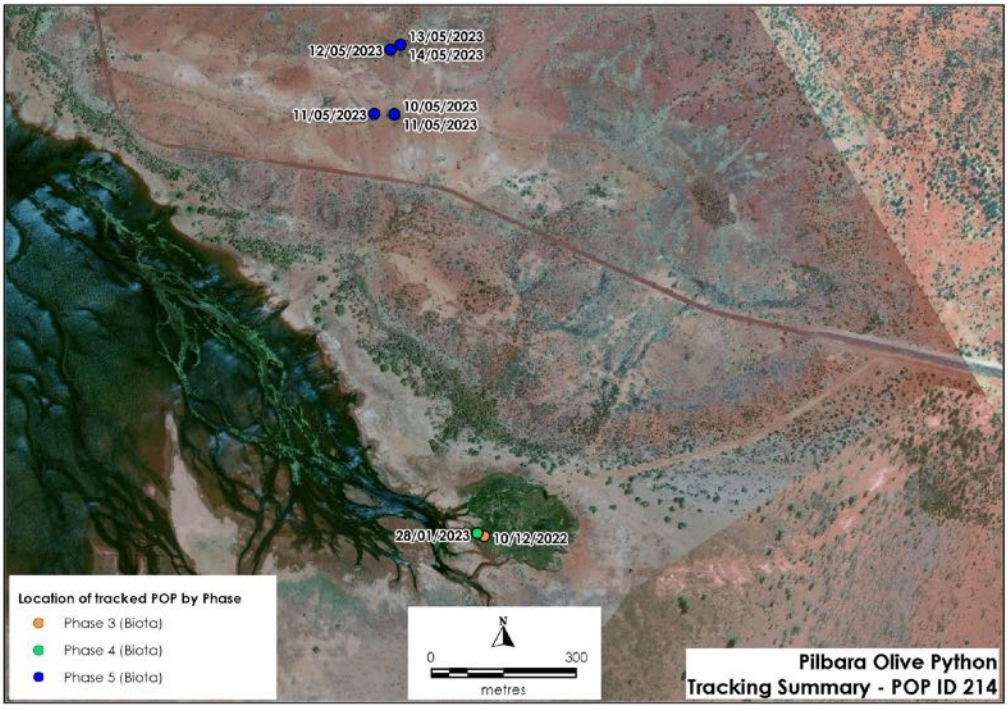

Figure Error! No text of specified style in document..**24. Records of radio-signal tracking and capture locations of POP 214 at Ophthalmia Dam.**

POP 215, an adult male, was first captured in phase 4 on 23/01/2022, in shallow water north of the Typha reedbed (Table 22). It was released on January 25 and tracked on January 28 and 29. On both occasions, it was triangulated within the Typha reedbed (see Figure **Error! No text of specified style in document.**.25). In the first three days of phase 5 (10-12 May 2023), it was relocated four times in the Typha reedbed. Due to high water levels, its position within the reedbed was not triangulated. On 13 May 2023, it was found 8m offshore N of the reedbed, stationary in 20cm of water with eyes and nostrils exposed (Plate 42), and was hand captured for health assessment (Plate 43). It was healthy, with surgical scars healing well, and at 6135g, had put on 600g since initial capture. On 14 May, it was released at point of capture.

Plate 42. POP 215, as found on 13/05/2023, stationary with eyes and nose exposed, 8m offshore in Ophthalmia Dam.

Plate 43. POP 214 midbody scales on 14/05/2023, showing the vibrant colours which can appear when POP are exposed to sun or torchlight.

Table 22. Known locations of POP 215.

Date	Location	Latitude	Longitude	Accuracy*	Comments
23/01/2022	North of Typha reedbed	-23.3519	119.8980	+/- 2m	Found in water north of Typha reedbed. Initial capture.
28/01/2023	Typha reedbed	-23.3525	119.8976	+/- 15m	Triangulated within Typha reedbed
29/01/2023	Typha reedbed	-23.3524	119.8977	+/- 15m	Triangulated within Typha reedbed
10/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
11/05/2023 12:45 hrs	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
11/05/2023 21:00 hrs	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
12/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
13/05/2023	8 m offshore, N of Typha reedbed	-23.3516	119.8994	+/- 2m	Found in 20cm deep water, 8m offshore, N of Typha reedbed. Appeared to be stationary, with just nose and eyes exposed above water. Hand captured for health assessment and released at same location the following day. Healthy, and has gained 600g.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

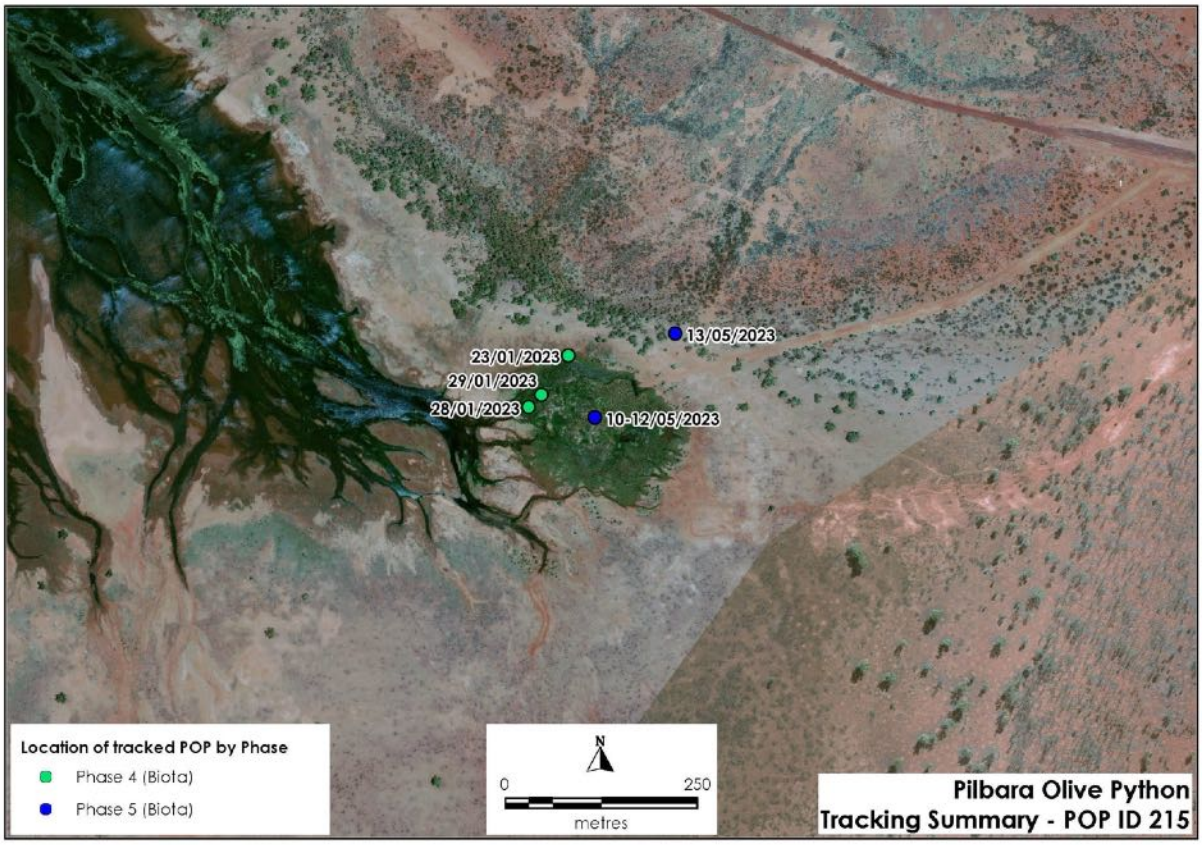

Figure Error! No text of specified style in document..**25. Records of radio-signal tracking and capture locations for POP 215 (Dec 2022 and Jan 2023) at Ophthalmia Dam.**

POP 217, an adult male, was first captured in phase 4 on 28/01/2023, at the base of a dead tree on dry ground south-west of the Typha reedbed (Figure **Error! No text of specified style in document.**.26, Table 23). It was fitted with a VHF transmitter and released on January 29. Throughout phase 5 (10-14 May 2023), its signal was detected offshore in the direction of the Typha reedbed. Due to high water levels, its position was not triangulated.

Table 23. Known locations of POP 217.

Date	Location	Latitude	Longitude	Accuracy*	Comments
28/01/2023	South west of Typha reedbed	-23.3550	119.8966	+/- 2m	Found beneath a dead tree on dry ground south-west of Typha reedbed. Initial capture.
10/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
11/05/2023 12:45 hrs	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
11/05/2023 21:00 hrs	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
12/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
13/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.
14/05/2023	Typha reedbed	-23.3526	119.8984	+/- 100m	In offshore Typha reedbed. Not triangulated; central GPS point of Typha reedbed provided.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

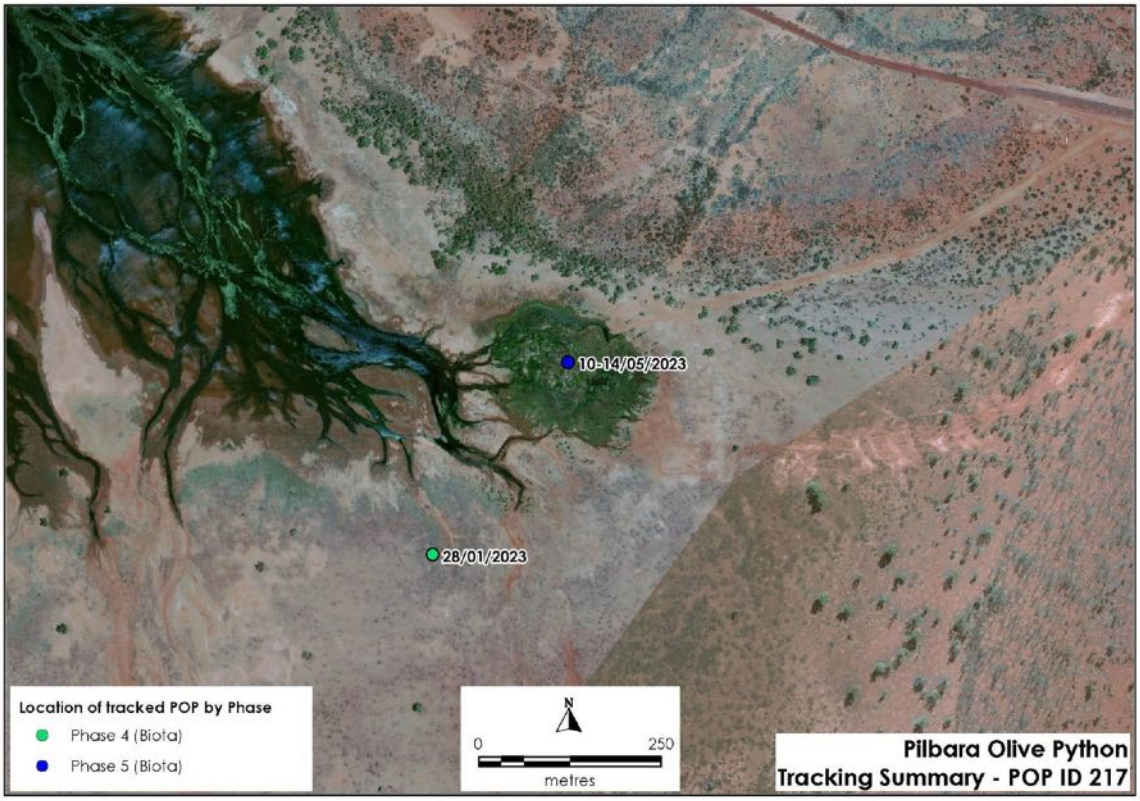

Figure Error! No text of specified style in document.**.26. Capture locations for POP 217 at Ophthalmia Dam.**

POP 218, an adult female, was first captured in phase 5 on 13/05/2023, next to an access track, 1.4km NW of the Typha reedbed, and 500m ESE of dam wall (Table 24, Figure **Error! No text of specified style in document.**.27). It was fitted with a VHF transmitter, released at point of capture and has not yet been tracked.

Table 24. Known locations of POP 218.

Date	Location	Latitude	Longitude	Accuracy*	Comments
13/05/2023	Access track, 1.4km NW of Typha reedbed	-23.3429	119.8894	+/- 2m	Found next to access track, 1.4km NW of Typha reedbed and 500m ESE of dam wall. Initial capture.

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

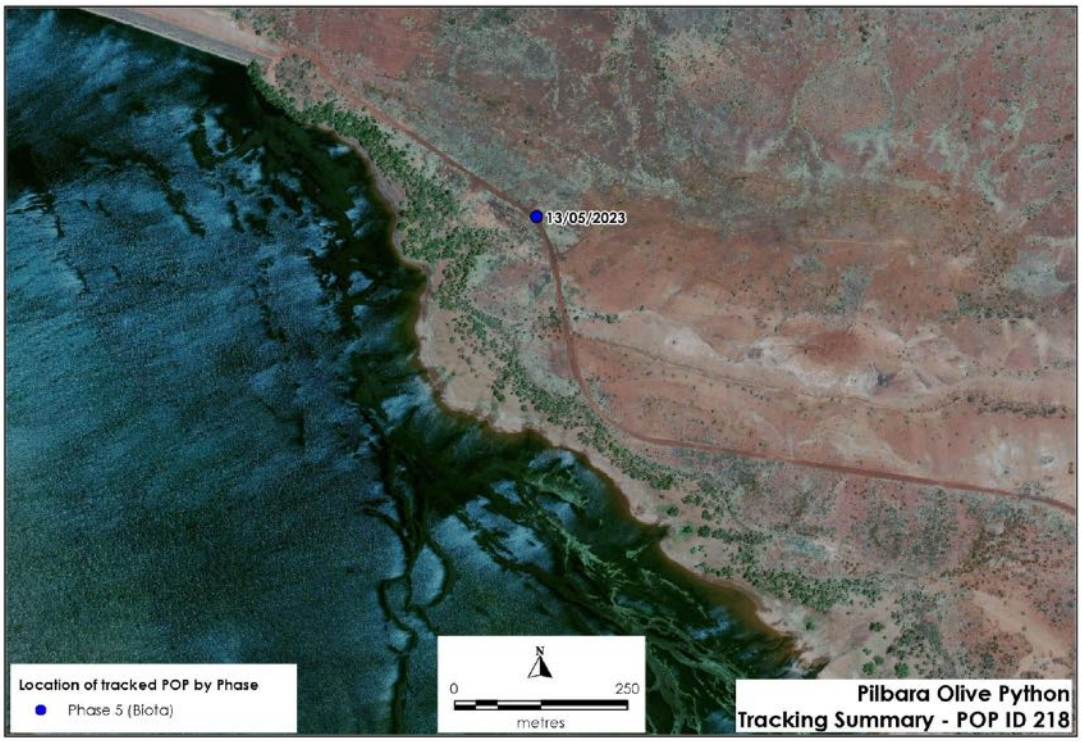

Figure Error! No text of specified style in document..**27. Capture location for POP 218 at Ophthalmia Dam.**

POP 222, a juvenile female, was first captured in phase 4 on 23/12/2022, on dry ground south of the Typha reedbed (Table 25). It was fitted with a VHF transmitter and released on January 25. On January 28, it was found without radio-tracking under a dead tree north of the Typha reedbed, elevated amongst raised branches (**Error! Reference source not found.**, Figure **Error! No text of specified style in document.**.28). As it appeared healthy and had been released only three days before, it was not captured for a health assessment. On January 29, it was radio-tracked, with position triangulated within the Typha reedbed. No signal was detected in Phase 5 (May 2023).

Table 25. Known locations of POP 222.

Date	Location	Latitude	Longitude	Accuracy*	Comments
23/01/2022	South of Typha reedbed	-23.3534	119.8981	+/- 2m	Found on dry ground south of Typha reedbed. Initial capture.
28/01/2023	North of Typha reedbed	-23.3518	119.89918	+/- 2m	Found beneath a dead tree, resting amongst raised branches.
29/01/2023	Typha reedbed	-23.3529	119.89898	+/- 15m	Triangulated within Typha reedbed

*Accuracy of +/- 2m indicates that we are confident of this location, and any inaccuracies are due to GPS error only.

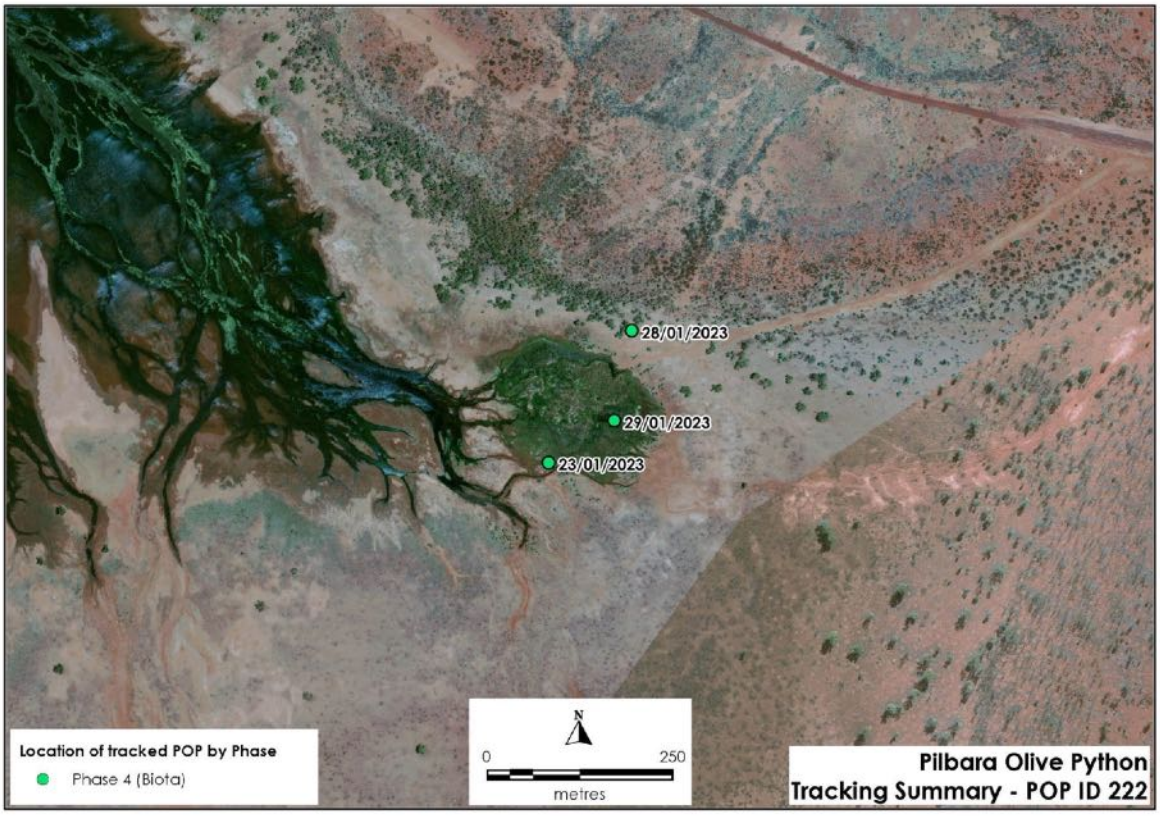

Figure Error! No text of specified style in document..**28. POP 222 radio-tracked locations at Ophthalmia Dam.**

Appendix 8

Helix Genetic Diversity Report

Helix
Molecular
Solutions

Population Genetics of Pilbara Olive Python

Western Ridge, Ophthalmia Dam, & Millstream 2022-2023

Prepared for BHP

September 2023

Author: S. McConnell, Z. Hamilton

Quality Checking History

Version: Peer review: R. Teale
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POP Population Genetics

Contents

Tables

Figures

1.0 Methodology

1.1.1 DNA Extraction

Scale clips removed when assigning pythons unique scale clip identification numbers were preserved and then stored in 100% ethanol and the temperature maintained at -20°C prior to DNA extraction. DNA was then extracted from 1-2 scales using the Qiagen DNeasy Blood & Tissue kit (QIAGEN) following the spin-column protocol for *purification of total DNA from animal tissues*. This methodology was modified slightly by performing the final elution step twice, using 70µl and 80µl of buffer AE. The resulting purified DNA was then stored at -20°C prior to use.

1.1.2 PCR procedures and microsatellite primer screening

Genomic DNA was mined for microsatellites using a MiSeq Illumina Next Generation Sequencing run (Peter Spencer, unpublished data). Twenty-five pairs of microsatellite primers were selected from this run, and a further twelve primer pairs were chosen based on published data by Ciavaglia, Dridan and Linacre (2017). The total thirty-seven primer pairs were screened for their ability to amplify the extracted DNA of the Pilbara olive python, *Liasis olivaceus barroni,* by performing a gradient temperature Polymerase Chain Reaction (PCR) to determine the optimal annealing temperature. Thirty primers pairs were subsequently retained owing to their ability to generate a stable PCR-product.

M13- tags were added to the 5'- end of all forward primers, following the protocol of Schuelke (2000). To facilitate multiplexing, M13- tags were also added to 6FAM, VIC, NED and PET fluorescent dyes as described by Venkatsen, Hauer & Rasgon (2007).

PCR amplification was performed on an Eppendorf Thermalcycler using the following procedure: an initial denaturation step of 95°C for 3 min, followed by 25-35 cycles of 95°C for 30s, annealing at 56-60°C (depending on the locus) for 1 min, extension at 72°C for 1 min, then (to facilitate M13 binding) a further 8 cycles of 95°C for 30s, annealing at 53°C and extension at 72°C for 1min. Lastly, there was a final extension step of 72°C for 5min. For Fragment Analysis (FA), 3ul of the amplified PCR-product was loaded with 15.5µl Hi-Di formamide (Applied Biosystems) and 0.13µl Genescan 500 LIZ (Thermo Fisher) internal size standard and run on an Applied Biosystems 3730xl DNA Analyser (ABI, Melbourne). When multiple PCR-reactions (each containing primers with different dye attachments) were loaded in the same well for FA, 5ul of each PCR reaction was first mixed and 3ul of the resulting mixture was loaded for FA. Fragments were then scored manually using Geneious V2023.0.3 software.

1.1.3 Molecular Analysis

Basic population genetic statistics were generated using *R* (R core team, 2022) software and the excel add-in GenAlEx 6.5 (Peakall and Smousse, 2006, 2012). The *R* package 'PopGenReport' (Adamack and Gruber, 2017) was used to assess data quality (percentage of missing data, null alleles), total number of alleles per site and private alleles. We determined the frequency of null alleles per locus using the method of Brookfield (1996). Departures from Hardy-Weinberg equilibrium (*HWE*) were assessed for each locus and population with the *R* package 'pegas' (Paradis, 2010), using an exact test with 1000 Monte Carlo permutations and $\alpha = 0.05$. GenAlEx was used to calculate the number of alleles (N_A), number of effective alleles (N_E) and Information index (I). The *R* package 'diveRsity' v1.9.90 (Keenan, 2017) was used to estimate observed and

expected heterozygosity's (H_O and H_E), resampled allelic richness (A_R) and the resampled inbreeding coefficient (F_{IS}), with confidence intervals for A_R and F_{IS} calculated using a bootstrap procedure (1000 randomizations) and α = 0.05. Resampling was used to correct for differences in sample size.

Population genetic structure was assessed in STRUCTURE v2.3.4 (Pritchard *et al*., 2000). The 'diveRsity' package was also used to evaluate genetic differentiation by estimating population pairwise F_{ST} (Weir & Cockerham, 1984) and G_{ST} (Nei & Chesser, 1983) values, with 95% confidence intervals calculated from a bias-corrected bootstrap method (1000 randomizations).

The 'adegenet' v1.3-1 package (Jombart and Ahmed, 2011) in *R* (R core team, 2022) was used to perform both a Principal Components Analysis (PCA) of POP populations and a Discriminant Analysis of Principal Components (DAPC) for an additional assessment of genetic structure.

2.0 Results

2.1 Genetic Diversity

All thirty microsatellite loci successfully amplified in each of the twenty-nine Pilbara olive python individuals used in this study, with a missing data rate of only 0.03 % (see Table 1). As in the 2022 study, eleven of the thirty loci remain monomorphic (holding only one allele) in all individuals and so were removed from further genetic analyses (Table 1), while all nineteen polymorphic loci were retained (Table 2Table 2). No null alleles were detected. Three loci showed significant deviations from HWE in the Ophthalmia Dam subpopulation (*F17, P2, F4*) however, as they were in equilibrium in the other two subpopulations they were not excluded from the analysis.

The information index (*I*) measures both allelic and genetic diversity and can be used to compare the informativeness of different loci. *F4, P16, P7* and *F22,* with mean values of 1.71 (± SE 0.11), 1.11 (± SE 0.12), 1.09 (± SE 0.16) and 1.08 (± SE 0.05) respectively, hold the highest values for *I* and are the most informative loci used in this study (Table 2). In contrast, the least informative loci are *P19, P22, P14* and *P3* with the lowest respective *I* values of 0.09 (± SE 0.09), 0.15 (± SE 0.15), 0.29 (± SE 0.18) and 0.30 (± SE 0.03). Loci with the highest and lowest values for *I* also contained the highest and lowest number of alleles and number of effective alleles, which is not surprising as both measures are a function of gene diversity.

The number of alleles (N_a) observed across the nineteen polymorphic loci ranged from two to thirteen (mean 2.93 ± SE 0.20; Table 2 and Table 3), with an average detection increase of 0.37 alleles relative to that observed in the 2022 collection (mean 2.56 ± SE 0.26). The mean number of effective alleles encompassed values from 1.05 (± SE 0.05, P19) - 4.73 (± SE 0.55, F4) (Table 3). The same four loci were identified as the most informative in 2022 and 2023, however there has been a change in one of the least informative loci from *P2* in 2022, to *P3* in 2023. This substitution is likely due to the mean increase of 0.18 effective alleles observed in the *P2* locus during this collection (N_e = 1.24 ± SE 0.15), while there was no change in the number of effective alleles observed in the *P3* locus (N_e = 1.18 ± SE 0.01).

All within-population descriptive diversity measures consistently identified the subpopulation at Ophthalmia Dam as more genetically diverse than either Millstream or Western Ridge, but only by a marginal amount (Table 4 and Table 5). Ophthalmia Dam holds the highest number of alleles (mean N_a= 3.26 ± SE 0.43), effective alleles (mean N_e = 2.44 ± SE 0.29) and private alleles (P_a = 11), as well as the highest values for allelic richness (mean A_r = 2.68 (95% CI 2.37 – 3.00)), observed and unbiased expected heterozygosity (mean H_o = 0.50 ± SE 0.07, uH_e = 0.49 ± SE 0.06), and the information index (mean *I* = 0.85 ± SE 0.20). Slightly depressed but comparable levels of diversity are observed in the Millstream (mean N_a= 2.74 ± SE 0.30, N_e = 2.00 ± SE 0.22, P_a = 7, A_R = 2.45 (95% CI 2.10 – 2.74), H_o = 0.47 ± SE 0.07, uH_e = 0.45 ± SE 0.06, *I* = 0.71 ± SE 0.10) and Western Ridge subpopulations (mean N_a= 2.79 ± SE 0.28, N_e = 1.90 ± SE 0.19, P_a= 5, A_R = 2.37 (95% CI 1.95 – 2.68), H_o = 0.39 ± SE 0.06, uH_e = 0.41 ± SE 0.06, *I* = 0.67 ± SE 0.10). Overlapping error bars for allelic richness indicate there are no significant differences in this measure between any of the sampled subpopulations, supporting the inference that observed levels of genetic diversity are broadly similar between the three Pilbara olive python subpopulations.

Parallel conclusions were made during the 2022 report, however here we observed higher levels of diversity in the Western Ridge subpopulation during this 2023 data collection. In 2022, the subpopulation at Western Ridge was identified as the least genetically diverse, however its genetic diversity values in 2023 are almost indistinguishable to those observed in the Millstream subpopulation (Table 4). Furthermore, the total number of private alleles detected has decreased from 25 (2022) to 23 (2023), with two more private alleles detected in the Ophthalmia Dam subpopulation, and four fewer private alleles observed in the Millstream subpopulation (Table 4). All between-year diversity differences can likely be attributed to sampling effect, whereby the larger number of samples collected (nine more in Ophthalmia Dam and four more in Western Ridge) allowed us to capture a more complete picture of the genetic diversity contained within the Pilbara olive python subpopulations, and alleles that previously went undetected in the Western Ridge and Ophthalmia Dam subpopulations have now been identified.

The inbreeding coefficient (F_{IS}) was low in all three subpopulations, with mean F_{IS} ranging from -0.15 (95% CI -0.40 - -0.11, Millstream) to -0.03 (95% CI -0.16 - 0.04, Ophthalmia Dam) (Table 4). Negative values such as this indicate that inbreeding within the subpopulations is rare and genetic diversity is high, as illustrated by a slight excess of heterozygotes in all subpopulations. Inbreeding estimates obtained in 2023 are slightly elevated relative to those observed in 2022, which is again likely due to the increased number of samples contributing to a better coverage of the population and the derivation of more reliable estimates.

Table 1. Summary information for 11 monomorphic loci used on three Pilbara subpopulations (Western Ridge, Ophthalmia Dam and Millstream) of *Liasis olivaceus barroni* (POP *n*=29).

T optimal annealing temperature, Bp allele size range, *Na* number of alleles, N_e effective number of alleles, SE standard error). Observed and expected heterozygosity (H_o & H_e), as well as the inbreeding coefficients (F_{IS}) were unable to be determined due to the lack of variation of these 11 loci across the three populations (Western Ridge, Ophthalmia Dam and Millstream).

Loci	Repeat bases	Primer sequences (5' - 3')	*T*	*Bp*	*Na ± SE*	*Ne ± SE*	I ± SE
MsF10_M13 (F10)	(GAAT)n	F: TGTAAAACGACGGCCAGTTGGACAGCCAGACAAATCAA	54, 56, 58, 60	165	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: CATGATATGGCTTGTCCATGA					
Lo_176796_M13 (P6)	(AC)13	F: TGTAAAACGACGGCCAGTTGACAATGATTCTGCCCGCC	56	187	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: CCCACCAAAGTCATCCACGA					
Lo_328094_M13 (P1)	(AC)12	F: TGTAAAACGACGGCCAGTACAGCATATAGGTTTCTCTGCAA	56, 58	108	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: ACCTGGTCAAATGTCAGGGT					
Lo_269966_M13 (P17)	(AC)11	F: TGTAAAACGACGGCCAGTTGGAAGCTTTGATATGGTCAAGGA	56	301	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: CCCTCTAGTTATCGCCTGCC					
MsF28_M13 (F28)	(TGATC)n	F: TGTAAAACGACGGCCAGTTGACTCAGAACTGTGCCTAATCC	58, 60	375	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: TCCATCTGAAACTTTGTTCCCT					
Lo_520782_M13 (P12)	(AC)17	F: TGTAAAACGACGGCCAGTGAGTAGGTGGTCAGAACAATGA	56	218	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: TTCCTGGGATGGGCTTCCTA					
Lo_135136_M13 (P20)	(AG)12	F: TGTAAAACGACGGCCAGTGCAATGACCCAATCAGCCTC	56	304	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: TGGCCACCAGGGTCTGTATA					
Lo_230365_M13 (P23)	(AC)12	F: TGTAAAACGACGGCCAGTTCACTAATTTATGCTATAGCCCAGT	58	364	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: TGAAGAAAGACTAGCTGCCCT					

Loci	Repeat bases	Primer sequences (5' - 3')	*T*	*Bp*	*Na* ± *SE*	*Ne* ± *SE*	I ± SE
Lo_208439_M13 (P5)	(AG)13	F: TGTAAAACGACGGCCAGTAGCCTGCTCTTAACTGTGCA	56	130	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: CTCAAGAGGACAAGAGTTCCC					
Lo_538461_M13 (P10)	(AC)11	F: TGTAAAACGACGGCCAGTGGGCTTTCTGTTAAATACACAGG	56	195	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: CTCACCTGATCCCTGACAGT					
MsF25_M13 (F25)	(CATC)n	F: TGTAAAACGACGGCCAGTTTGAGGATCTCATCAACTCCG	54, 56, 58, 60	328	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
		R: TAGTGAGTGGAACATGGTCTCTTG					

Table 2. **Information and genetic diversity statistics generated from 19 polymorphic loci used on three Pilbara subpopulations (Western Ridge, Ophthalmia Dam and Millstream) of *Liasis olivaceus* subsp. *barroni* (POP *n*=29).**

T optimum annealing temperature (°C), *bp* allele size range, N_a average number of alleles, N_e effective number of alleles, *I* information index. ± values indicate the standard error.

Loci	Repeat bases	Primer sequences (5' - 3')	*T*	*Bp*	N_a ± *SE*	N_e ± *SE*	I ± *SE*
MsF5_M13 (F5)	(TAGA)n	F: TGTAAAACGACGGCCAGTAGCTGCCCAAAGTTGCTATG R: TTCTCCTTCAGGTTCAGCTTG	54, 56, 58, 60	165-185	3.33 ± 0.33	2.27 ± 0.23	0.95 ± 0.04
MsF3_M13 (F3)	$(ATGA)_n$	F: TGTAAAACGACGGCCAGTCGTAGGGCTGGTTGGTTTTA R: CAAGCCTAAGCTGACAAGCA	54, 56, 58, 60	168-184	3.67 ± 0.33	2.29 ± 0.12	0.99 ± 0.02
MsF9_M13 (F9)	$(TATC)_n$	F: TGTAAAACGACGGCCAGTTGGGTGGGAAATAGCTGAAG R: CCTGTAAACTGCCCAGAGTTG R: TTCTCCTTCAGGTTCAGCTTG	54, 56, 58, 60	182-186	2.00 ± 0.00	1.65 ± 0.25	0.53 ± 0.14
MsF17_M13 (F17)	$(GATA)_n$	F: TGTAAAACGACGGCCAGTTGCAATATTGTCATAATTCAACCC R: ACTGATTCACTTGGAGGCCC	58	367-375	3.00 ± 0.00	2.56 ± 0.39	0.98 ± 0.11
Lo_308896_M13 (P11)	$(AC)_{15}$	F: TGTAAAACGACGGCCAGTTGGGTCAAAGCAAACCACCT R: AGCTTTGATGCTGAAGGGCA	56	209-223	2.33 ± 0.33	2.11 ± 0.47	0.74 ± 0.19
MsF27_M13 (F27)	$(ATCT)_n$	F: TGTAAAACGACGGCCAGTCAAACCCTTTCCCAAATTTCTC R: CTCATGACCAGCCAGGTCTC	54, 56, 58, 60	399-411	2.67 ± 0.33	1.75 ± 0.41	0.65 ± 0.19
MsF4_M13 (F4)	$(CTTT)_n$	F: TGTAAAACGACGGCCAGTTTGCTTGTCACATTTACAGGG R: CCTTCCATTGCTCAGTCCTT	60	180-224	7.33 ± 0.88	4.73 ± 0.55	1.71 ± 0.11
Lo_468641_M13 (P3)	$(ATC)_{14}$	F: TGTAAAACGACGGCCAGTTGCTCCTAAAGAAGAAACTGGGT R: ACTTTGGACAAACACAATCACAA	58	123-129	2.33 ± 0.33	1.18 ± 0.01	0.30 ± 0.03
MsF16_M13 (F16)	$(CATT)_n$	F: TGTAAAACGACGGCCAGTGCAAAGGACCTTGGAAGAAA R: GCTTTATGGTGATAACCAGCACT	58	374-386	3.33 ± 0.33	2.59 ± 0.61	0.98 ± 0.22
Lo_232191_M13 (P22)	$(AG)_{16}$	F: TGTAAAACGACGGCCAGTTGGAGACTTGAGGTTCACAGA R: TGCTGTCCCTTCCTCATCCT	56	355-359	1.33 ± 0.33	1.13 ± 0.13	0.15 ± 0.15
Lo_200246_M13 (P13)	$(AC)_{18}$	F: TGTAAAACGACGGCCAGTACTCAGATGCACGTTTCCCA	56	220-226	2.33 ± 0.33	1.90 ± 0.42	0.64 ± 0.23

Loci	Repeat bases	Primer sequences (5' - 3')	*T*	*Bp*	N_a ± *SE*	N_e ± *SE*	I ± *SE*
		R: GGAGGGAGGGCTGGGATTT					
Lo_434196_M13 (P15)	$(AAT)_{16}$	F: TGTAAAACGACGGCCAGTCCCAAGCTAGGTCCGAATGG	56	230-245	3.67 ± 1.20	2.86 ± 0.94	1.03 ± 0.32
		R: ACGGCTGCTACATCTGAACA					
Lo_455916_M13 (P21)	$(AC)_{15}$	F: TGTAAAACGACGGCCAGTGGACAACTTCTTCCCAATCTCCA	56	330-332	2.00 ± 0.00	1.58 ± 0.21	0.51 ± 0.13
		R: GGATATGCCGCACCACAGAA					
Lo_284906_M13 (P7)	$(AGAT)_{13}$	F: TGTAAAACGACGGCCAGTGCACCACTGTCACTTGAGGT	56	181-201	4.00 ± 0.58	2.64 ± 0.57	1.09 ± 0.16
		R: TTAACTCAGGCCACCAGTGC					
Lo_125128_M13 (P19)	$(AC)_{10}$	F: TGTAAAACGACGGCCAGTACAGCAACTTGCCTATGGACT	56	304-306	1.33 ± 0.33	1.05 ± 0.05	0.09 ± 0.09
		R: CTGGCCATACTGACCAGCAA					
Lo_133106_M13 (P2)	$(AGC)_{12}$	F: TGTAAAACGACGGCCAGTTGGGATTGGCATGATTGGGT	58	105-123	2.00 ± 0.58	1.24 ± 0.15	0.31 ± 0.18
		R: CCTGGCCTGGGTACTTCC					
Lo_272924_M13 (P16)	$(ATC)_{13}$	F: TGTAAAACGACGGCCAGTCCTGGTCTTCATTTGTCTGCA	58	297-309	3.67 ± 0.33	2.78 ± 0.39	1.11 ± 0.12
		R: CCCAAAGTGCTAGTTTGTCATGG					
Lo_13018_M13 (P14)	$(ACCT)_{13}$	F: TGTAAAACGACGGCCAGTATGACATCAGAACCGCCTTT	56	230-238	1.67 ± 0.33	1.29 ± 0.22	0.29 ± 0.18
		R: AGATCACAGACCAGTTGGCA					
MsF22_M13 (F22)	$(ATCC)_n$	F: TGTAAAACGACGGCCAGTAGTGGCTGGACCAATGAGAT	54, 56, 58, 60	370-382	3.67 ± 0.33	2.63 ± 0.16	1.08 ± 0.05
		R: TTTGCCAAACACAGAGGACC					
Mean					**2.93 ± 0.20**	**2.11 ± 0.14**	**0.74 ± 0.06**

Table 3. **Total number of alleles detected in each of the 19 polymorphic loci, averaged across the three Pilbara subpopulations (Western Ridge, Ophthalmia Dam and Millstream) of *Liasis olivaceus* subsp. *barroni.***

H_o Observed heterozygosity, *uHe* Unbiased expected heterozygosity, F_{IS} inbreeding coefficient (within the subpopulation). 95% confidence intervals are displayed in brackets for $F_{IS.}$ Mean values are displayed with ± standard error.

Locus	F3	F9	F5	F17	P11	F27	F4	P3	F16	P22	P13	P15	P21	P7	P19	P2	P16	P14	F22
# of alleles	4	2	5	3	4	4	13	3	4	2	3	6	2	6	2	3	5	2	4

Table 4. **Genetic diversity of the three Pilbara subpopulations (Millstream, Ophthalmia Dam and Western Ridge) of *Liasis olivaceus* subsp. *barroni*.**

N_a number of alleles, N_e effective number of alleles, A_r allelic richness, *I* information index, P_a private alleles. Parentheses contain 95% confidence intervals. Mean is defined with ± standard error.

	Millstream			Ophthalmia Dam			Western Ridge		
Marker	H_o	uH_e	F_{is}	H_o	uH_e	F_{is}	*Ho*	uH_e	F_{is}
F3	0.5	0.56	0.03 (-0.46 - 0.43)	0.41	0.61	0.31 (-0.06 - 0.63)	0.43	0.62	0.25 (-0.33 - 0.75)
F9	0.83	0.53	-0.71 (-1 - -0.33)	0.47	0.47	-0.03 (-0.48 - 0.43)	0.14	0.14	-0.08 (-0.27 - -0.08)
F5	0.5	0.62	0.12 (-0.60 - 0.70)	0.71	0.64	-0.13 (-0.46 - 0.18)	0.57	0.5	-0.24 (-0.56 - -0.12)
F17	0.5	0.71	0.23 (-0.50 - 0.71)	0.65	0.69	0.03 (-0.38 - 0.35)	0.57	0.47	-0.30 (-0.75 - -0.12)
P11	0.33	0.3	-0.20 (-0.50 - -0.09)	0.82	0.69	-0.24 (-0.53 - 0.02)	0.29	0.53	0.42 (-0.40 - 1.0)
F27	0.83	0.67	-0.36 (-0.76 - -0.03)	0.29	0.26	-0.17 (-0.36 - -0.06)	0.29	0.28	-0.12 (-0.37 - -0.08)
F4	1	0.88	-0.24 (-0.64 - -0.20)	0.88	0.84	-0.08 (-0.31 - 0.08)	0.86	0.78	-0.18 (-0.56 - 0.07)
P3	0.17	0.17	-0.09 (-0.33 - -0.09)	0.18	0.17	-0.07 (-0.18 - -0.03)	0.14	0.14	-0.08 (-0.27 - -0.08)
F16	0.33	0.32	-0.14 (-0.46 - -0.09)	0.71	0.68	-0.07 (-0.41 - 0.23)	0.83	0.77	-0.18 (-0.64 - 0.14)
P22	0.33	0.3	-0.20 (-0.50 - -0.09)	0	0	0	0	0	0
P13	0.67	0.67	-0.09 (-0.71 - 0.46)	0.12	0.11	-0.06 (-0.17 - -0.03)	0.43	0.54	0.14 (-0.75 - 0.71)
P15	0.5	0.41	-0.33 (-0.71 - -0.09)	0.94	0.81	-0.20 (-0.35 - -0.08)	0.71	0.6	-0.27 (-0.78 - 0.29)

Commented [1]: Although the Millstream population hasn't changed since 2022 (no new samples), Allelic richness values are slightly different because because it is calculated using the resampling method, which corrects for differences in population size. Because the size of the other populations has increased, resampling has caused the Millstream value to increase

	Millstream			Ophthalmia Dam			Western Ridge		
Marker	H_o	uH_e	F_{is}	H_o	uH_e	F_{is}	*Ho*	uH_e	F_{is}
P21	0.33	0.49	0.25 (-0.50 - 1.00)	0.41	0.45	0.06 (-0.42 - 0.55)	0.14	0.14	-0.08 (-0.27 - -0.08)
P7	0.5	0.56	0.027 (-0.50 - 0.43)	0.81	0.76	-0.11 (-0.38 - 0.12)	0.71	0.56	-0.37 (-0.75 - -0.20)
P19	0	0	0	0	0	0	0.14	0.14	-0.08 (-0.27 - -0.08)
P2	0.17	0.17	-0.09 (-0.33 - -0.09)	0.18	0.36	0.49 (-0.17 - 1.00)	0	0	0
P16	0.67	0.55	-0.33 (-0.71 - -0.14)	0.71	0.69	-0.05 (-0.36 - 0.23)	0.57	0.75	0.18 (-0.34 - 0.59)
P14	0	0	0	0.47	0.43	-0.13 (-0.55 - 0.33)	0.14	0.14	-0.08 (-0.27 - -0.08)
F22	0.83	0.67	-0.36 (-0.76 - -0.03)	0.71	0.68	-0.07 (-0.44 - 0.25)	0.43	0.63	0.26 (-0.31 - 0.75)
Mean	**0.47 ± 0.07**	**0.45 ± 0.06**	**-0.15 (-0.40 - -0.11)**	**0.51 ± 0.07**	**0.50 ± 0.06**	**-0.04 (-0.17 - 0.03)**	**0.39 ± 0.06**	**0.41 ± 0.06**	**-0.05 (-0.29 - 0.02)**

2.2 Genetic Differentiation

F_{ST} (Weir & Cockerham 1984) and G_{ST} (Nei & Chesser 1983) are both measures of subpopulation-level genetic differentiation that range from 0 (subpopulation allele frequencies are identical, no differentiation) to 1 (subpopulations are fixed for different alleles, completely distinct from one another). An F_{ST} range of 0 – 0.05 is considered to indicate low genetic differentiation, 0.05 – 0.15 is moderate, 0.15 – 0.25 is high, and values greater than 0.25 are considered to suggest a very high degree of differentiation. G_{ST} is better suited to multiallelic microsatellite data while F_{ST}, which was originally formulated for biallelic data, is the most widely referenced measure. To account for the values of each, we report both estimates (Table 5).

F_{ST} and G_{ST} presented respective global values of 0.1618 and 0.1173 (data not shown), indicating that 83.82% and 88.27% of the variance in allele frequencies is due to intra-(within) population genetic variation. Pairwise comparisons reveal a moderate to high F_{ST} ranging from 0.1395 - 0.1885 (Table 5). Of the three POP subpopulations, Western Ridge and Ophthalmia Dam are the most genetically comparable with the lowest F_{ST} estimate of 0.1333, while Millstream appears to be the most distinct subpopulation, with high F_{ST} values of 0.1730 (Western Ridge) and 0.1851 (Ophthalmia Dam) (Table 5). Pairwise G_{ST} values are slightly lower (range 0.0740 – 0.1036) and show the same population trends as that observed with F_{ST} (Table 5). In concordance with the observed similarities in genetic diversity, overlapping confidence intervals indicate that no significant differences in population differentiation were detected between any of the populations, nor either of the methods (F_{ST}/G_{ST}) used.

Pairwise F_{ST} and G_{ST} estimates are slightly lower here than that reported in 2022, however the same general trend of least - most differentiated populations, and lack of significant differences across both populations and estimation methods is observed. Error bars for estimates obtained across years also overlap, illustrating that there are no significant differences in the magnitude of pairwise population differentiation measured with F_{ST} and G_{ST} between the 2022 and 2023 collections.

Table 5. Pairwise population differentiation measured as G_{ST} (Nei & Chesser 1983) and F_{ST} (Weir & Cockerham 1984), estimated for the three Pilbara subpopulations (Millstream, Ophthalmia Dam and Western Ridge) of *Liasis olivaceus* subsp. *barroni*.
G_{ST} below diagonal, F_{ST} above diagonal. Brackets represent 95% confidence intervals generated by bootstrapping 10,000 times.

	Millstream	Ophthalmia Dam	Western Ridge
Millstream	-	0.1851 (0.1045 – 0.2652)	0.1730 (0.1059 – 0.2448)
Ophthalmia Dam	0.1036 (0.0550 – 0.1538)	-	0.1322 (0.0753 – 0.1968)
Western Ridge	0.0938 (0.0555 – 0.1358)	0.0740 (0.0413 – 0.1144)	-

2.3 Population Genetic Structure

2.3.1 STRUCTURE

STRUCTURE (Pritchard *et al*., 2007) is a popular Bayesian model-based clustering method that determines the minimum number of subpopulations (K) required to explain the total sum of within-population genetic variation. Based on the corresponding values of K (populations number) there is an obvious inflection pattern at K= 4 (Figure 1),

indicating that amongst the total 29 sample POP individuals, the molecular data suggest four genetic sub-populations.

The STRUCTURE analysis revealed these four genetic clusters correspond to cluster 1: the entire Millstream subpopulation, cluster 2: six out of the seven individuals from Western Ridge, while clusters 3 and 4 consist of a split of Ophthalmia Dam individuals along with the extra Western Ridge individual (Figure 47). Cluster 3 consists of five Ophthalmia Dam individuals and one Western Ridge individual, and cluster 4 contains 11 Ophthalmia Dam individuals. This result encompasses an additional two clusters identified in 2023 relative to 2022. These extra clusters are also observed in the large spread and opposing directions of Ophthalmia Dam individuals and the single Western Ridge individual that approaches the boundaries of Ophthalmia Dam in the PCA scatterplot (Figure 3 and Figure 4), and illustrate the way population boundaries can change as more genetic information is collected.

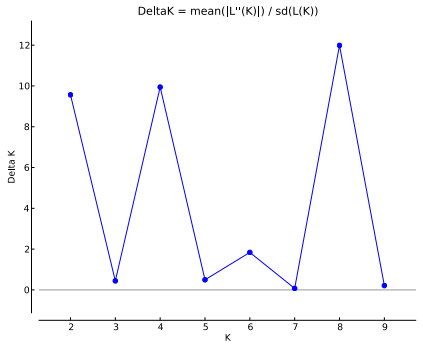

Figure 1. Identification of the number of populations best fitting the polymorphic loci (n=19) for the *Liasis olivaceus* subsp. *barroni* data (n=29) from the three sampling locations (Western Ridge, Ophthalmia Dam and Millstream).

Plot of the mean log probability of *K* (population n=umber) for number of populations 1-10 over ten runs of each.

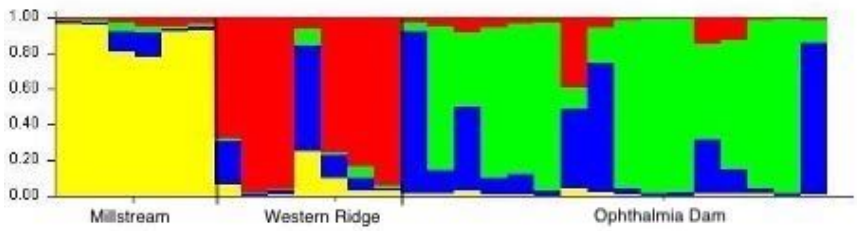

Figure 2. Structure plot of *Liasis olivaceus* subsp. *barroni* from the three sampled subpopulations (Western Ridge, Ophthalmia Dam and Millstream).

Each vertical bar represents a single individual, population codes indicated along the X-axis (Millstream, Western Ridge, Ophthalmia Dam), whilst the proportion of ancestry components in an individual in relation to other populations is depicted along the Y axis.

2.3.2 Principal Components Analysis (PCA) of POP populations

Principal Components Analysis (PCA) is a multivariate analysis method that is often used as a model-free alternative to STRUCTURE. The overall genetic variability that exists among individuals is summarised into principal components (PC's), with the first PC accounting for the largest variance.

A PCA of the Pilbara olive python individuals separated all three subpopulations along the first and second axes, with one Ophthalmia Dam individual appearing to belong to the Western Ridge subpopulation (Figure 3). The third axis highlights the amount of variation in the Ophthalmia Dam individuals and clusters the subpopulations at Millstream and Western Ridge together (see Figure 4). Such a result seems counterintuitive given the results from other differentiation analyses (e.g. Table 5, Figure 1, Table 6) that highlight Millstream as a unique population, and may indicate the second axis lacks sufficient information to reliably discern genetic trends.

The PCA's generated in 2022 show a larger separation of all subpopulations along the first and second axes, and the first and third axes cluster the Western Ridge and Ophthalmia Dam subpopulations together while Millstream remains distinct. The closer clustering of the different subpopulations to one another along with the larger spread of individuals in the Ophthalmia Dam and Western Ridge subpopulations that is observed in this study may reflect the way increased genetic information can blur the population definitions as allele frequency variations within a population become more apparent, and differences between population appear less stark.

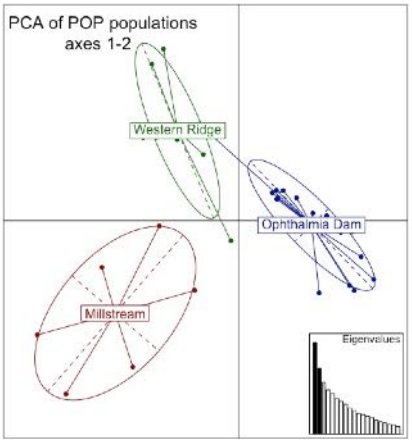

Figure 3. **Principal Components Analysis using data from 19 polymorphic microsattelite loci genotyped for 29 *Liasis olivaceus* subsp. *barroni*. individuals collected from three Pilbara subpopulations (Millstream, Ophthalmia Dam and Western Ridge), axes 1 and 2.**

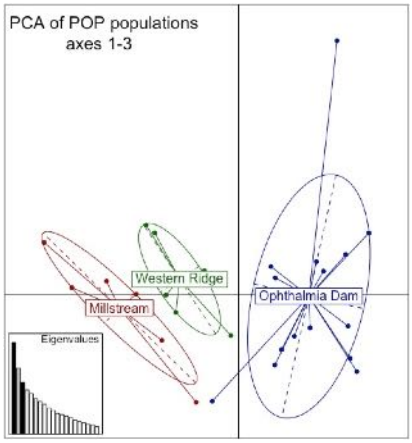

Figure 4. **Principal Components Analysis using data from 19 polymorphic microsatellite loci genotyped for 29 *Liasis olivaceus* subsp. *barroni*. individuals collected from three Pilbara subpopulations (Millstream, Ophthalmia Dam and Western Ridge), axes 1 and 3.**

2.3.3 Discriminant Analysis of Principal Components (DAPC)

A Discriminant Analysis of Principal Components (DAPC) was performed as an additional test of group assignment. DAPC incorporates aspects of both STRUCTURE and PCA: it is a multivariate method that using *K* -means clustering of principal components to identify groups. Individuals are discriminated into groups through the segregation of genetic variation into between- and within-groups components, maximising the between-groups variation.

As in STRUCTURE, the DAPC analysis identified four clusters and confirmed 100 % assignment of individuals to the Millstream subpopulation, along with the single Western Ridge individual that is reassigned to the Ophthalmia Dam subpopulation (Table 6). Interestingly, the Ophthalmia Dam subpopulation that this Western Ridge individual is assigned to appears to be different in the DAPC analysis relative to that identified by STRUCTURE. A possible explanation is that this individual holds genetic diversity values that are marginal between the two identified subpopulations at Ophthalmia Dam, as seen by the way its data point sits at approximately the midpoint between the opposing directions of Ophthalmia Dam individuals when viewed along the first and second axes of the PCA (Figure 3).

As in the 2022 study, the subpopulation at Millstream remains distinct. Like the conclusions drawn for the PCA and STRUCTURE analyses, the extra clusters and split observed in Ophthalmia Dam and Western Ridge is likely due to the increase in genetic samples creating a more complex and complete picture of the within- and between-groups partitioning of genetic variation.

Table 6. Assignment of *Liasis olivaceus* subsp. *barroni* individuals to defined populations based on the microsatellite data.

Individual from OpD source population assigned to WR population indicated by '*'

	Millstream	Western Ridge	Ophthalmia Dam + Western Ridge	Ophthalmia Dam
Millstream	6	0	0	0
Western Ridge	0	6	1*	0
Ophthalmia Dam	0	0	10	6

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Appendix 9

Helix Genetic Relatedness Report

Helix
Molecular
Solutions

Pilbara Olive Python Relatedness

Western Ridge, Ophthalmia Dam, & Millstream 2022-2023

Prepared for BHP

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Report Title

Contents

1.0 Methodology

The genetic relatedness between a pair of individuals (dyad) is estimated as the probability that two alleles (at a particular locus, one drawn randomly from each individual) are identical by descent (IBD). Simply, these alleles have originated from a common ancestor (Jacquard 1972; Blouin 2003; Wang 2022). The degree of relatedness between two individuals is estimated relative to a pre-defined reference population using the coefficient of relatedness, *Rxy*, which measures the expected fraction of alleles within the genome of these individuals (x,y) that are shared (by descent). This is estimated and based on a reference population containing alleles at frequencies found in the population sampled (Milligan 2003; Blouin 2003; Attard *et al.* 2018).

Dyads are classified and divided into discrete genealogical categories according to their percentage of IBD alleles. Jacquard's (1972) 9 condensed identity states, S_1-S_9, fully describe the way 4 alleles (2 from diploid individual X and 2 from diploid individual Y) can be partitioned at a locus. As the states cannot be directly observed they must be ascertained based on probabilities derived from Jacquard's (1972) condensed IBD coefficients, Δ_1 - Δ_9, and defined relative to the reference population allele frequencies (Weir *et al.* 2006; Wang 2022).

Parent-offspring and full-sibling pairs are referred to as first-degree relatives and they will, on average, share one IBD allele per locus and have a predicted *Rxy* = 0.5 (Blouin 2003). Although the *Rxy* value is the same, the different allele sharing patterns for full-siblings relative to parent-offspring allows the distinction of these relationship classes according to their Δ_7 and Δ_8 values. More distant relationships can also be inferred, such as second-degree relative pairs (e.g. half-siblings, grandparents-grandchildren, avuncular) where there is an expected average *Rxy* = 0.25 (Blouin 2003), and so on. However, as the relationship becomes more distant the number of possible allele sharing patterns and thus sampling variance associated with the estimate increases (Blouin 2003), and values estimated with microsatellite markers are typically only approximate for the dyad (Taylor 2015).

1.1 Selection of the best estimator

Several relatedness estimators are available, however it is well established that their performance is heavily influenced by various population characteristics, such as the demographic history and true relatedness composition (Csilléry et al. 2006, Taylor 2015), and the diversity and allele frequency distribution of the genetic markers used (Blouin 2003). For these reasons, no single estimator performs optimally under all scenarios and it is therefore important that simulations be conducted to inform selection of the most appropriate estimator and the likely reliability of estimates obtained (Wang 2011a, Taylor 2015).

In this analysis, we used the *related* v1.0 package (Pew *et al.* 2014), which is an *R* implementation of the widely referenced *COANCESTRY* program (Wang 2011a), to conduct simulations and estimate the pairwise genetic relatedness of all individuals sampled in this study. We simulated 500 pairs of individuals for each degree of relatedness (parent-offspring, full-sibling, half-sibling, unrelated) based on the allele frequency data, and estimated *Rxy* values with five method-of-moments estimators: QuellerGt (Queller and Goodnight 1989), LynchLi (Li *et al.* 1993), Ritland (Ritland 1996), LynchRd (Lynch and Ritland 1999) and Wang (Wang 2002). Since allele frequencies will differ if a population is structured into differentiated subpopulations, we ran separate simulations using reference allele frequencies for the entire population pooled ('Entire') and repeated the simulations for each of the subpopulations detected at Millstream, Western Ridge and Ophthalmia Dam. In acknowledgement of the population divisions detected with the

STRUCTURE analysis, we also calculated relatedness using allele frequencies generated by subdividing the subpopulations into their four *STRUCTURE* identified groups, however as no new relationships were detected at this smaller sampling scale, we chose to retain the original site groupings to increase sample size.

The performance of all five relatedness estimators relative to the “true” relatedness value was compared in terms of bias, precision and error. Bias and precision were jointly assessed with the root mean square error (RMSE) using the approach suggested by Wang (2007), the relative variability of the data in relation to the mean is analysed with the coefficient of variation (CV), which was selected following the work of Taylor (2015) to permit comparability of datasets with different mean values. The standard deviation is reported instead of CV for unrelated dyads only, as CV is not suitable for use with negative values. Misclassification rates among the simulated relationship categories of parent-offspring, full-sibling, half-sibling and unrelated were calculated using the method of Blouin (1996), whereby the midpoint between the mean values of any two simulated sampling distributions is used as the threshold to classify dyads into different relationship categories, and error rates are calculated as the proportion of dyads of a given relationship that were classified as another relationship. The type I error is defined as the percentage of misclassified individuals falling to the right of the distribution (less related individuals classified as more related), and the type II error encompasses those falling to the left of the distribution (more related individuals classified as less related, Blouin 1996). Lastly, we calculated Pearson’s correlation coefficient (*r)* to evaluate goodness-of-fit between the observed and expected relatedness values.

1.2 Construction of relatedness networks

Pairwise relatedness values were calculated using the optimal estimator of *related* v1.0, with 95% confidence intervals estimated by bootstrapping over loci 10,000 times. Values were designated into the genealogical categories of first-degree, second-degree or unrelated according to the pre-determined population-specific threshold values, and first-degree dyads were further divided into parent-offspring or full-sibling based on their Δ_7 and Δ_8 values. To avoid problems associated with the inclusion of false positives and loss of information due to the exclusion of false negatives, we separately report results for dyads whose lower 95% confidence interval does, and does not, encompass 0.

The relationship between individuals was visualised spatially using the program *Gephi* (Bastian *et al.* 2009). A network of relationships was plotted using individuals as nodes and relatedness values as edge thicknesses. The plug-in *GeoLayout* was used to represent nodes according to their geographic sampling location. We then superimposed the network on a map using the program *Inkscape* (Inkscape Project 2020).

2.0 Results

The Wang (2002) estimator was used to calculate pairwise relatedness values for all *Liasis olivaceus* subsp. *barronii* individuals sampled across the Pilbara, and the data was incorporated into a geographic relatedness network. This approach was used to identify and characterise the level of gene flow that occurs between and within each of the three study sites.

The Wang (2002) estimator was selected as simulation results indicate it provided the best fit for our data. Wang (2002) is well suited to variable microsatellite markers and is unbiased when used on small sample sizes (Wang 2017). This estimator held the highest correlation between observed and expected relatedness values as measured by Pearson's *r*, and the lowest root mean squared error and coefficient of variation values across the largest percentage of simulated relationship categories (Appendix Table 1). The Wang (2002) estimator also represented the lowest percentage of misclassification errors for the majority of relatedness categories, and misclassification errors remain similar when the entire population or each of the subpopulations are used as the allele frequency reference (Appendix Table 2). Misclassification errors between the first-degree relationship categories (full-siblings, parent-offspring) and unrelated individuals are low and range from 0 - 6.4%, however, misclassification errors between adjacent relationship categories (e.g. half-sibling and all other categories) average 23%, so results reported for the second-degree relationship level must be viewed with caution.

When all Pilbara olive pythons used in this study were treated as sourced from a single population, genetic relatedness (*Rxy*) ranged from -0.738 – 0.731, with an average of -0.082 (±0.251 SD) indicating that the majority of sampled individuals were genetically unrelated to one another (Figure 1). When subpopulations are analysed separately, mean relatedness values are broadly similar and retain the signature of no genetic relationship. The lowest mean *Rxy* is found at Western Ridge, with an average of -0.142 (+/- SD 0.334). This subpopulation also has the largest range, with values from -0.817 – 0.550. *Rxy* of the Millstream subpopulation ranged from -0.659 to 0.627, with an average of -0.091 (+/- SD 0.319). Average relatedness is marginally higher in the Ophthalmia Dam subpopulation, where *Rxy* ranges from -0.661 to 0.488 and averages -0.030 (+/- SD 0.239). These results make sense when viewed alongside the negative F_{IS} values (see Helix report "Population Genetics of Pilbara Olive Python: Western Ridge, Ophthalmia Dam, & Millstream 2022-2023"), which indicate a large, diverse and outbred population. Note, negative results for *Rxy* occur when the reference allele frequencies are estimated from the same sample that is being analysed and indicate that individuals are less related than the average expectation (Wang 2017).

2.1 Stringent dataset: Confidence intervals do not intersect 0

To avoid false positive results (i.e. classifying unrelated individuals as related), we only consider individuals to be related when the lower 95% confidence interval of the pair of individuals (dyad) does not encompass 0, and the *Rxy* value is higher than the threshold for unrelated individuals (for threshold values see Appendix Table 3). As this is a conservative approach that may include false negatives, we also separately report results for dyads that hold *Rxy* values above the threshold value for unrelated and have a lower confidence interval that intersects 0 (see "Relaxed dataset: Confidence intervals intersect 0").

As exemplified by the low mean values, all Pilbara olive python populations consist of mostly unrelated individuals. The relatedness distribution is dominated by values consistent with the first-degree relationship category and smaller populations contain a higher total percentage of related individuals (Figure 1).

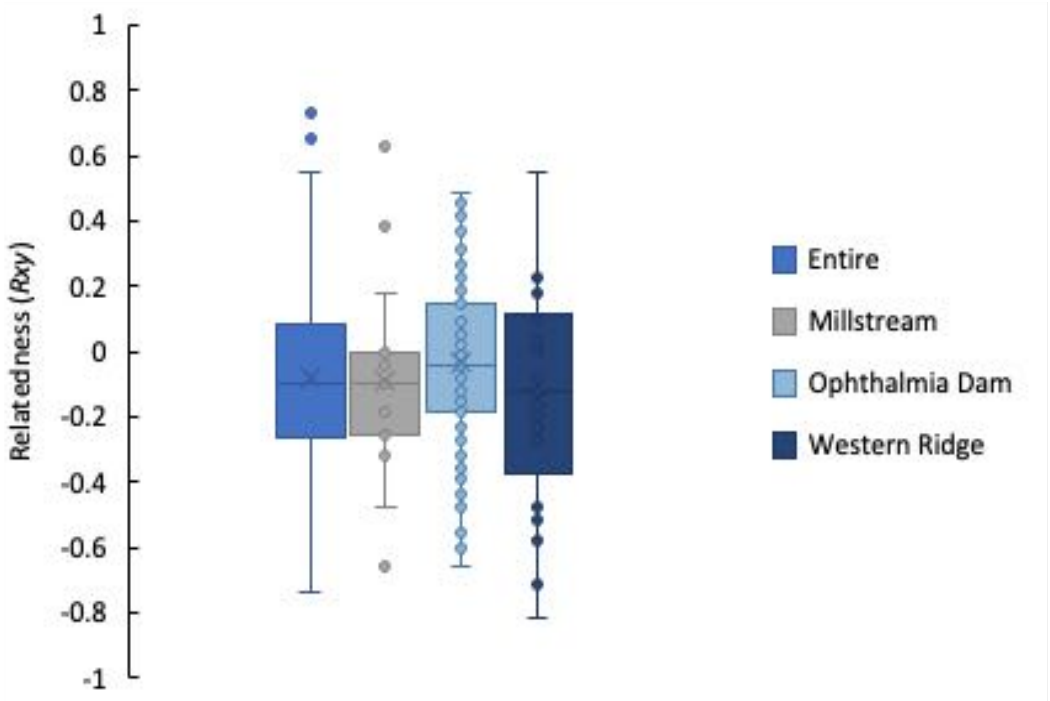

Figure 1: The distribution of mean relatedness values (x) for each of the three subpopulations (Millstream, Ophthalmia Dam, Western Ridge) and all collected *Liasis olivaceus* subsp. *barronii* specimens (Entire).
Boxes represent the upper and lower quartiles, divided by the median. The 10 and 90 percent quartiles are depicted by lines, dots represent the outliers.

When the three subpopulations were analysed in isolation, Millstream contains the largest percentage of related individuals (Figure 1). *Rxy* values consistent with the level of first-degree relationship (one parent-offspring, one full-sibling dyad) are detected in 13% (2/15) of dyads analysed (Figure 1, Figure 2). Paired specimens POP 101 and POP 103, and POP 105 and POP 104 each represent one related dyad (Figure 2, Appendix Table 4).

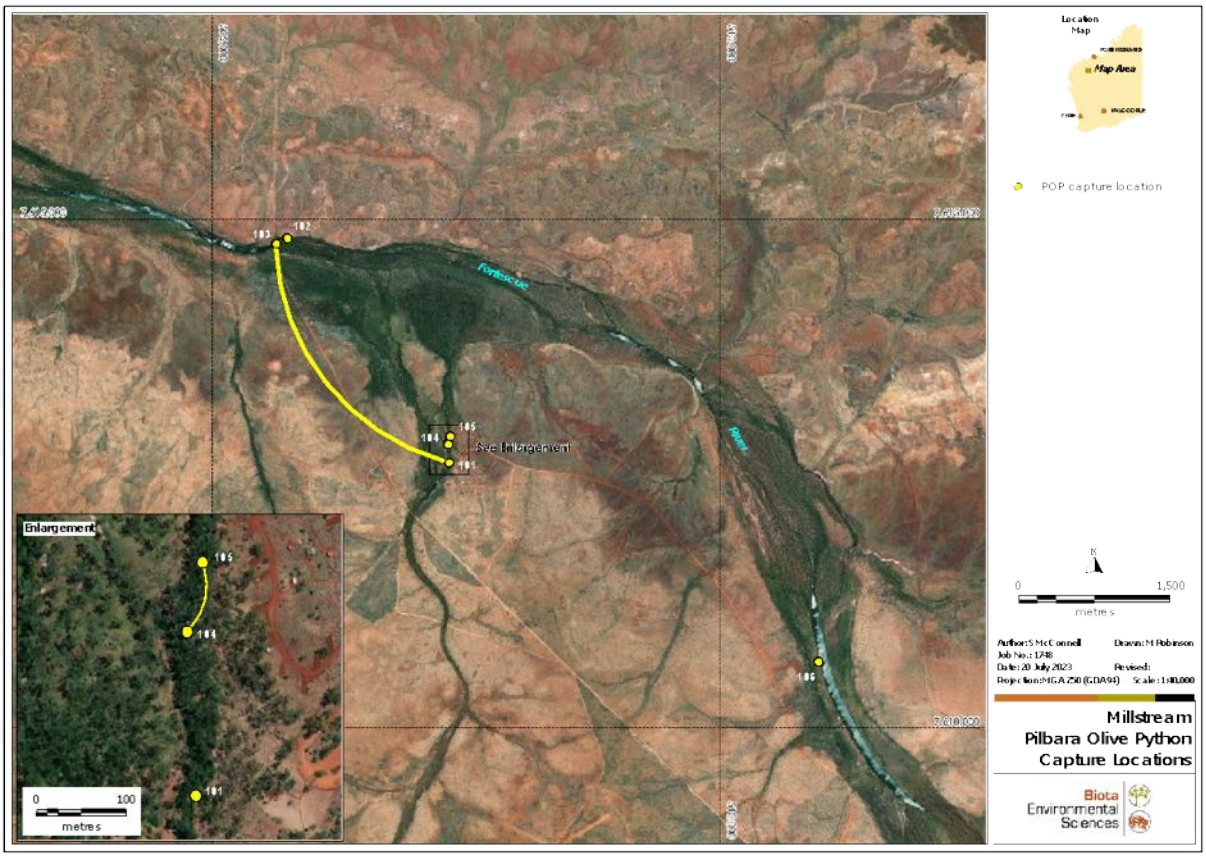

Figure 2: Map of Millstream project area plotting relatedness networks with values above 0.1235675 (threshold for unrelated individuals), for dyads whose lower 95% confidence interval does not intersect 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

Relative to the other two subpopulations, Western Ridge supports an intermediate percentage of related individuals. 10% (2/21) of all dyads analysed from the Western Ridge subpopulation are related to one another (Figure 1). 5% (1/21) of dyads are highly related accordant to the first-degree level (one parent-offspring dyad) and another 5% (1/21) are moderately related to the level of second-degree (Figure 1, Figure 3). Individual QR17 contributes to both identified dyads (Figure 3, Appendix Table 4).

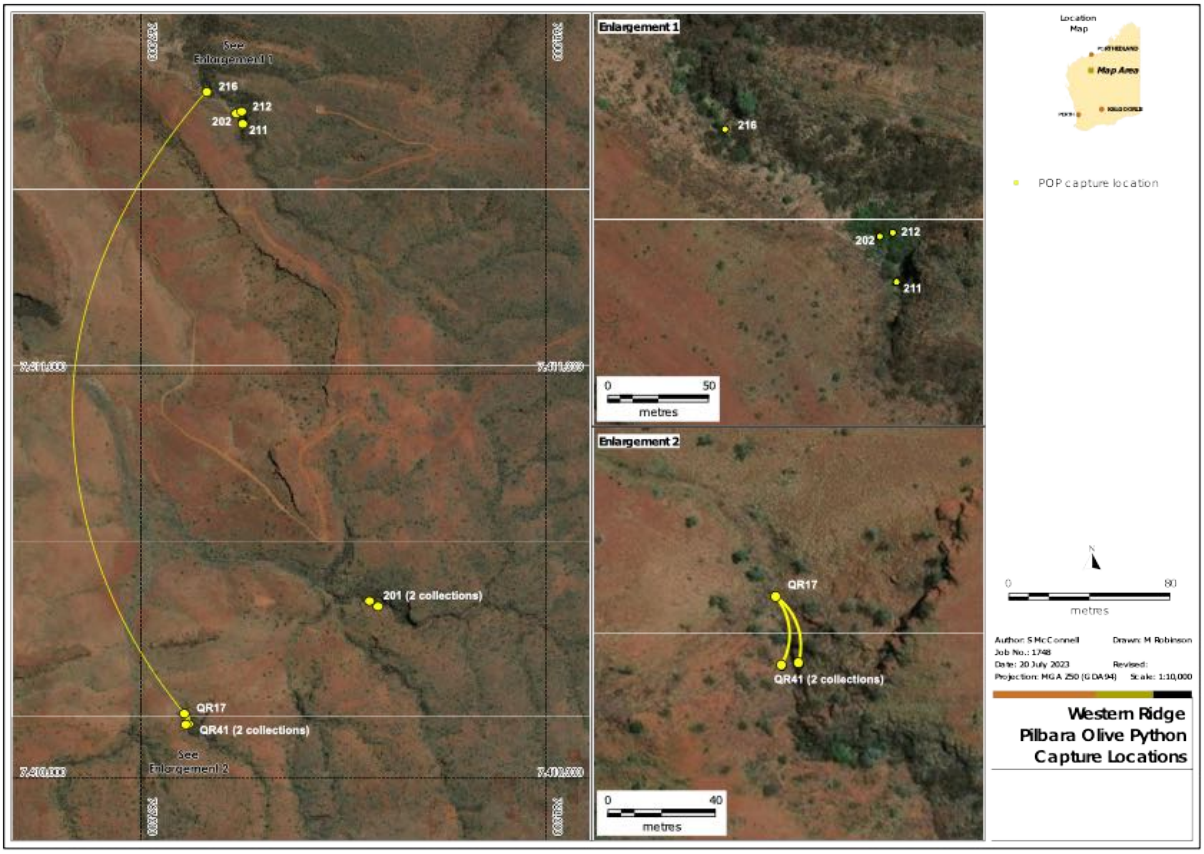

Figure 3: Map of Western Ridge project area plotting relatedness networks with values above 0.111620 (threshold for unrelated individuals), for dyads whose lower 95% confidance interval does not intersect 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

A slightly lower percentage (8%, 10/120) of related pairs are found at Ophthalmia Dam relative to the other two study sites (Figure 1). 6% (7/120) of all dyads hold values attributable to the level of first-degree relationships (four parent-offspring, three full-sibling dyads), and a further 3% (3/120) are moderately related with values indicative of the second-degree level (Figure 1, Figure 4). POP 210 and POP 213 represent the most connected individuals, with each detected in three dyads (Figure 4, Appendix Table 4).

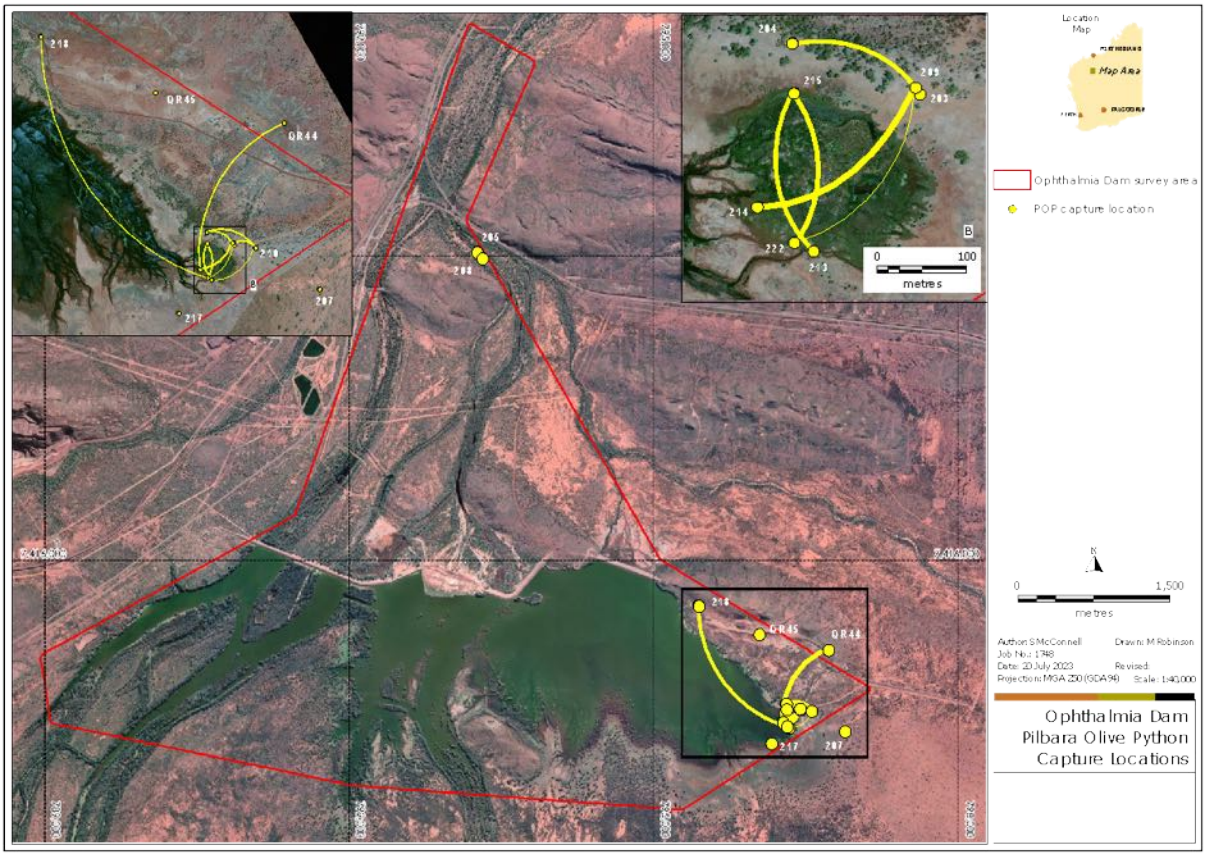

Figure 4: Map of Ophthalmia Dam project area plotting relatedness networks with values above 0.125131 (threshold for unrelated individuals), for dyads whose lower 95% confidance interval does not intersect 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

When allele frequencies for the entire population are used in estimating genetic relatedness, the reference point is set back in time and both recent and historical (i.e. before the population became subdivided) gene coalescences contribute to the final relatedness value (Wang 2011b). For this reason, relatedness estimates are always higher than when the subpopulation-specific allele frequencies are used, as in this latter case only recent coalescences (i.e. that occurred within the subpopulation) are relevant to the estimation procedure (Wang 2011b). Therefore, it may not be appropriate to use discrete relationship categories such as first- and second-degree and we will instead refer to the connected dyads in terms relating to the magnitude of observed relationship.

When considering the entire population of pooled individuals, the majority (96%, 392/406) of specimen pairs are unrelated to one another (Figure 1). Only a small proportion of the population (~3% or 11/406) were highly related and ~0.5% (2/406) hold moderate-low relatedness values (Figure 1). Individuals with the highest number of genetic connections are QR17 and QR13 (scale clip 210, who are each identified in three dyads (Appendix Table 4). Relationships between individuals are detected within study sites (Ophthalmia Dam: eight dyads, Western Ridge: three dyads, Millstream: two dyads), but not between sites – suggesting the existence of a significant historical barrier to gene flow (Figure 5). This barrier may be due to distance (i.e. isolation by distance, whereby genetic differences increase with geographical distance due to decreased dispersal events, Wright 1943), landscape features (i.e. isolation by resistance, where various aspects of the landscape can impede dispersal, Zeller *et al.* 2012), or behaviour. The lack of population connectivity observed here underpins the differentiation results (See

Population Genetics of POP, Table 5, Table 6, Figure 2-4), which suggest all three study sites are genetically unique and support independent subpopulations.

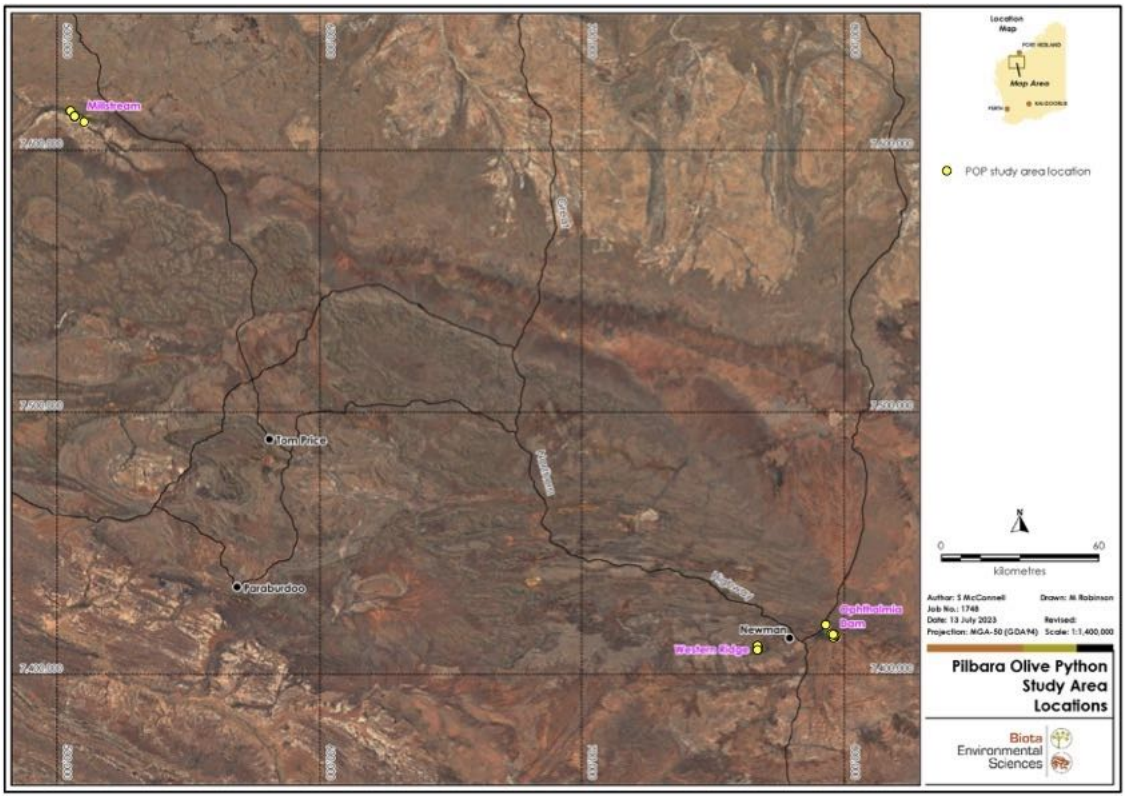

Figure 5: Map of entire project area plotting relatedness networks with values above 0.1241097 (threshold for unrelated individuals), for dyads whose lower 95% confidence interval does not intersect 0.

Liasis olivaceus subsp. *barronii* specimens are depicted by circles (nodes). Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found, however scale precludes the visualisation of relationships within study areas.

2.2 Relaxed dataset: Confidence intervals intersect 0

When model stringency was relaxed and all dyads with a relatedness value above the unrelated threshold were considered, many more connections become apparent - largely at the level of second-degree relatives. It is important to view these results with caution due to the inherent variability in IBD status and thus large variance and misclassification errors associated with relatedness estimation from more distantly related individuals (Appendix Table 2).

Due to the large increase in identified second-degree relationships, the percentage of second-degree and total related dyads is elevated for all populations (Figure 6). Millstream remains the subpopulation with the highest percentage of first-degree dyads, however the largest total proportion of related dyads is now detected at the Ophthalmia Dam and Western Ridge subpopulations, and to a lesser extent at the Millstream subpopulation and the pooled population with almost equal values (Figure 6).

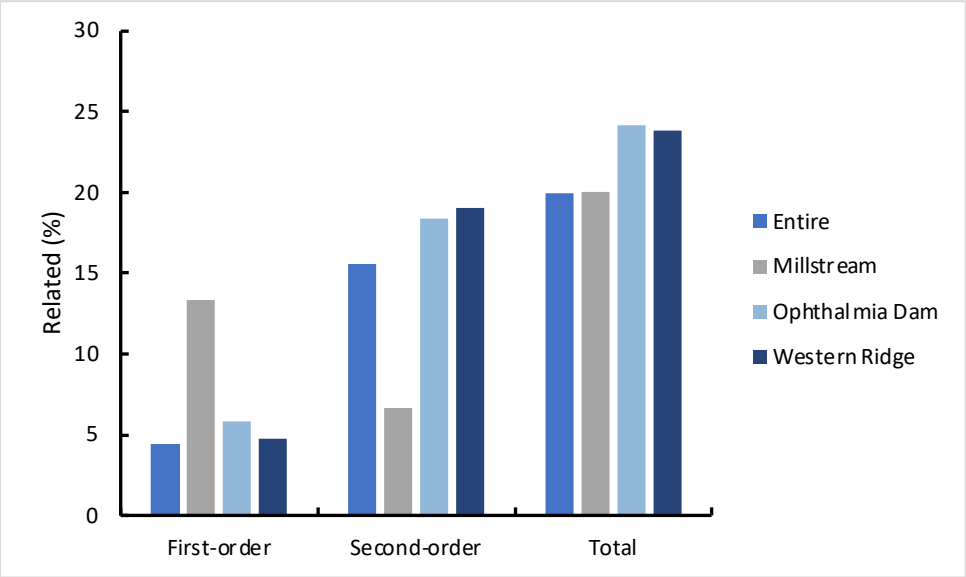

Figure 6: Percentage of relatedness values that fall within each relationship category, found in the three subpopulations, Millstream, Ophthalmia Dam and Western Ridge, and the entire population of *Liasis olivaceus* subsp. *barronii*, including dyads with confidence intervals that intersect zero.
Relationship categories are determined using population-specific threshold values.

When subpopulations were analysed separately, patterns of relatedness are detected at the lower second-degree level only. One more relationship is identified at Millstream (Figure 7), 16 at Ophthalmia Dam (Figure 9) and three at Western Ridge (Figure 8), bringing the overall percentage of related dyads at each subpopulation to 20%, 24% and 24%, respectively (Figure 6).

Genetically important individuals may be regarded as those who share genes with a large proportion of the population. At Millstream, POP 205 is connected to two dyads (Figure 7, Appendix Table 5) while all other related specimens are linked to only one other individual.

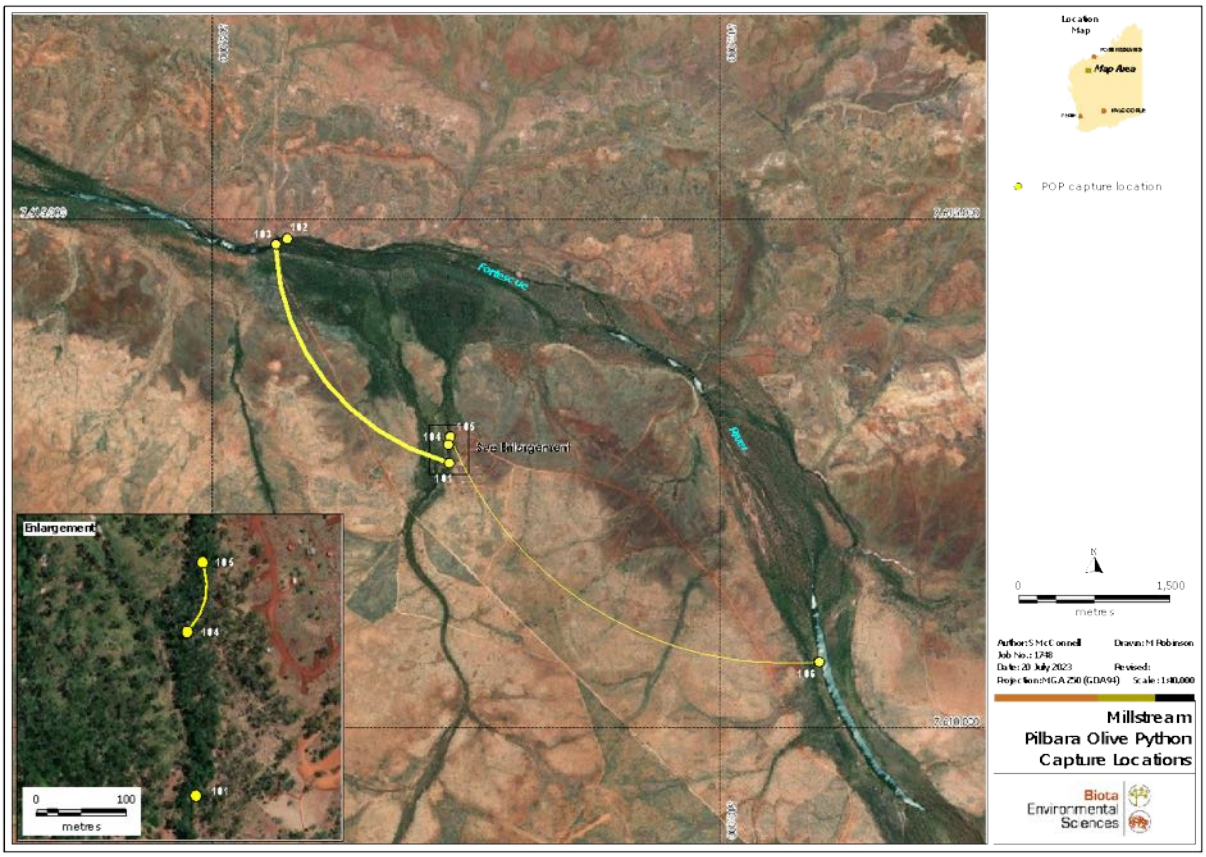

Figure 7: Map of Millstream project area plotting relatedness networks with values above 0.1235675 (threshold for unrelated individuals), including dyads whose lower 95% confidence interval intersects 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

POP 202 and QR17 are linked to a large amount of gene flow within the Western Ridge subpopulation, with each identified in three of the five detected relationships (Figure 8, Appendix Table 5).

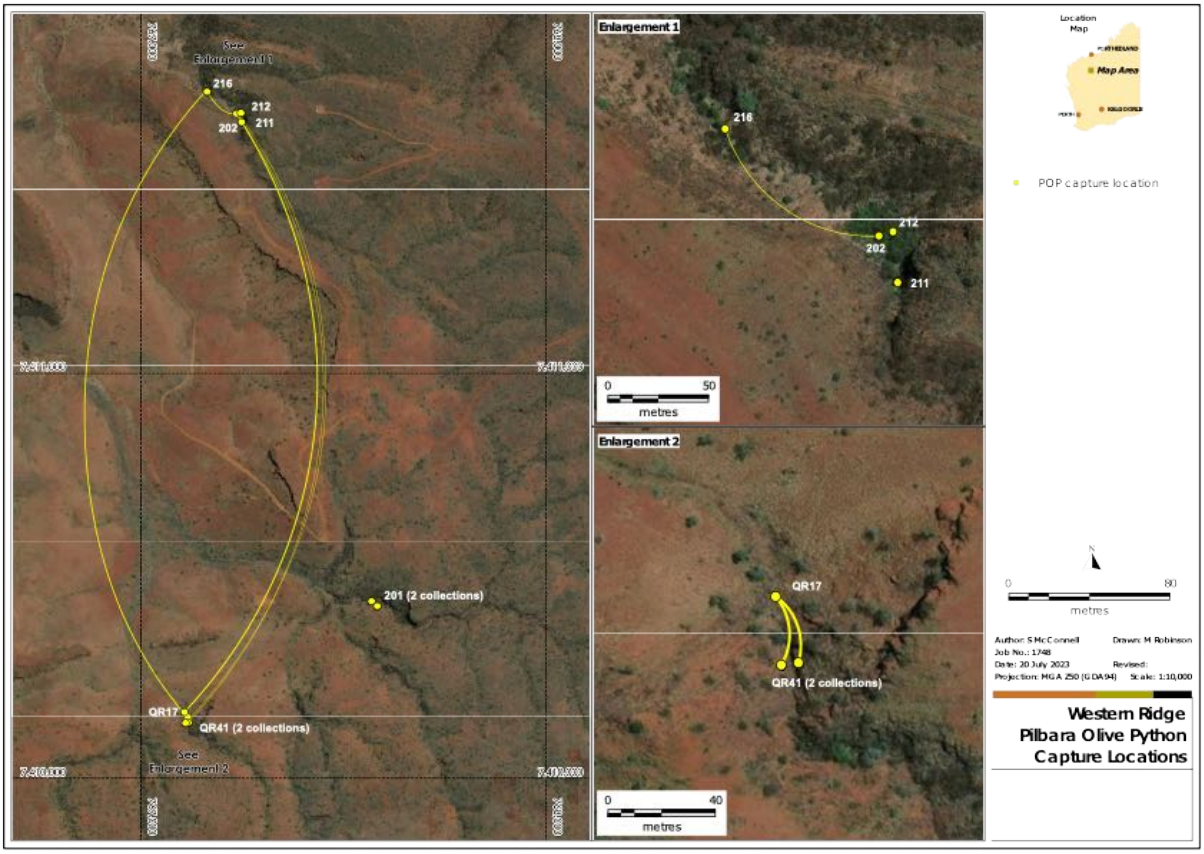

Figure 8: Map of Western Ridge project area plotting relatedness networks with values above 0.111620 (threshold for unrelated individuals), including dyads whose lower 95% confidance interval intersects 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

POP 210 contributes to the largest number of relationships at Ophthalmia Dam, with connections to eight other dyads (Figure 9, Appendix Table 5). POP 204, POP 203, POP 208 and POP 214 also represent genetically important individuals within the Ophthalmia Dam subpopulation, each with connections to five other dyads.

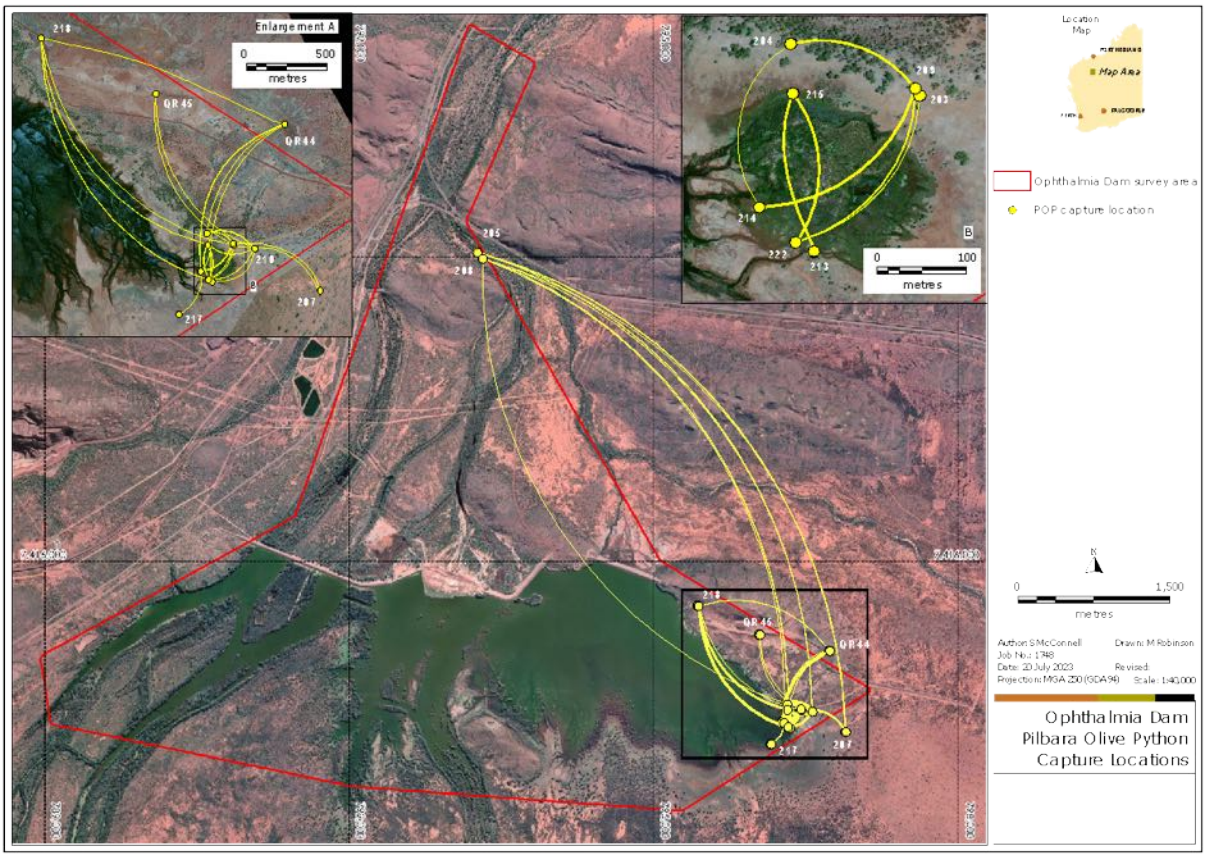

Figure 9: Map of Ophthalmia Dam project area plotting relatedness networks with values above 0.125131 (threshold for unrelated individuals), including dyads whose lower 95% confidence interval intersects 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

When the entire population is considered, one fifth (81/406) of all dyads share relatedness values indicative of a relationship. 4% (18/406) are highly related and 16% (63/406) are more distantly related (Figure 6). Most relationships are within sites, however, there appears to be some gene flow between all three sites which may represent contemporary dispersal events or historical population connections. Strong relationships occur purely within sites and candidate dispersers are all connected to one another with lower-level relatedness values only (Figure 10, Appendix Table 5).

Of the individuals with relatedness values above the unrelated threshold, at least two genetic relatedness connections are identified for each one (Appendix Table 5). The individual sharing the highest number of genetic relationships with other members of this study is POP 204 from Ophthalmia Dam, with links to 10 different individuals. Likewise, the Ophthalmia Dam individuals POP 203, POP 208, POP 213 and POP 214 each share eight connections with other sampled individuals, mostly within their own study site (Appendix Table 5). The Millstream individual POP 105 is identified in eight different relationships, and almost half of these are shared with individuals from Western Ridge (Appendix Table 5). This may suggest that POP 105 and its relatives are the progeny of individuals that dispersed and contributed genes across the two study sites.

Although dispersal between study sites is suggested to be a rare event, the greatest between-site proportion of genetic relatedness connections exist between the subpopulations at Western Ridge and Ophthalmia Dam (eight dyads), and Western Ridge and Millstream (six dyads, Figure 10). Only one relationship was detected between Ophthalmia Dam and Millstream, suggesting that there may be some landscape

resistance precluding dispersal between these sites, or sampling effect has impaired the identification of more relationships (Figure 10).

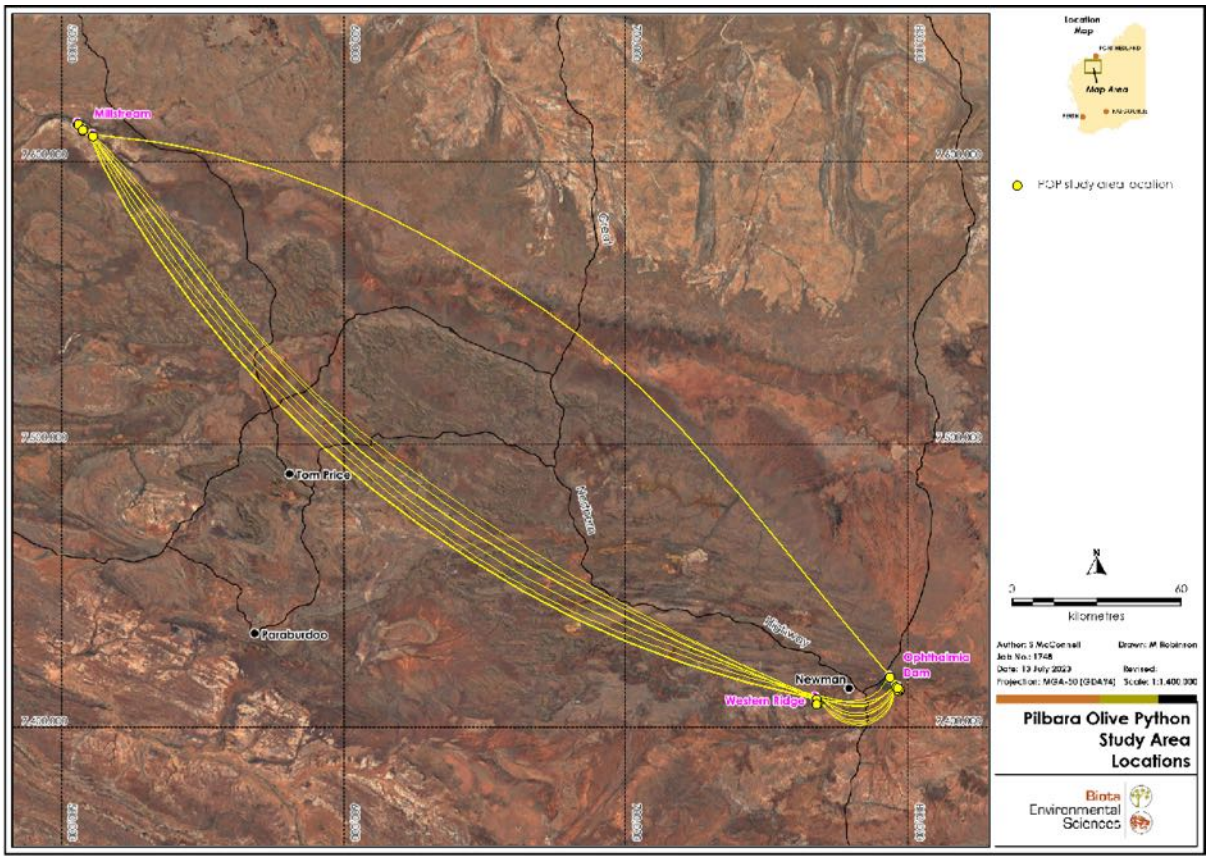

Figure 10: Map of entire project area plotting relatedness networks with values above 0.1241097 (threshold for unrelated individuals), including dyads whose lower 95% confidance interval intersects 0.

Curved lines (edges) connect *Liasis olivaceus* subsp. *barronii* specimens (nodes) for which a relationship was found. The thickness of edges is proportional to the relatedness value of the connected specimens, where a thicker line represents a higher relationship value.

When viewed in combination with the low inbreeding and moderate-high genetic diversity values, the large percentage of unrelated dyads detected despite the relatively isolated nature of the study sites is encouraging for the persistence of this species, as it suggests population boundaries are large and individuals are dispersing into each subpopulation from outside the study areas. It is interesting that of the related individuals, a large proportion of dyads occur in clusters with a few individuals contributing to many connections. This may suggest that the large number of unrelated individuals is due to small sample sizes, whereby an insufficient percentage of the population has been sampled to facilitate accurate characterisation of the population relationship composition. Continued sampling will be useful to determine the true relatedness composition of the populations and assess whether long-term survival may be impacted due to low dispersal between sites.

Studying the genetic relatedness of Pilbara olive python populations furthers understanding on the dispersal patterns and gene flow dynamics of this elusive species and facilitates the identification of genetically important populations to prioritise for conservation actions. Knowledge of dispersal patterns can help identify barriers to movement - information that is particularly important in the management of small, fragmented populations since isolation can lead to inbreeding depression and reduced population fitness through the loss of beneficial alleles (Escoda *et al*. 2017). A sampling

design that involves sampling at incrementally greater distances out from the survey sites may assist in the identification of barriers to dispersal (i.e. where relatedness sharply declines) and the detection of a ‘source’ population (i.e. one with many related links to other populations) that is crucial to the maintenance of gene flow equilibrium of this species.

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Appendix 1

Relatedness Information

Table 1: Pearson's correlation coefficient (*r*), root mean square error (RMSE), mean and coefficient of variation (CV) values for the relatedness estimates produced by five method of moment estimators: Wang (Wang 2002), LynchLi (Li *et al.* 1993), LynchRd (Lynch and Ritland 1999), Ritland (Ritland 1996), and QuellerGt (Queller and Goodnight 1989), over 4 relatedness categories (Parent-Offspring, Full-Sibs, Half-Sibs, Unrelated) using simulated pairs of known relatedness.

	Pearson's *r*	Parent-Offspring			Full-Sibs			Half-Sibs			Unrelated		
		RMSE	Mean	CV	RMSE	Mean	CV	RMSE	Mean	CV	RMSE	Mean	SD
Wang	0.79455	0.09683	0.49842	0.19424	0.15184	0.50548	0.30020	0.16472	0.25448	0.64703	0.19010	-0.00627	0.19000
LynchLi	0.78931	0.11158	0.49593	0.22483	0.15064	0.50745	0.29649	0.17280	0.25500	0.67736	0.19334	-0.01011	0.19308
LynchRd	0.77893	0.14923	0.49436	0.30165	0.18545	0.49947	0.37129	0.17406	0.24943	0.69782	0.13257	-0.00209	0.13256
Ritland	0.56504	0.29247	0.48439	0.60293	0.36666	0.51019	0.71841	0.26138	0.24449	1.06885	0.14454	-0.01440	0.14382
QuellerGt	0.78893	0.12199	0.49051	0.24795	0.15659	0.50125	0.31240	0.17154	0.24998	0.68622	0.17908	-0.01356	0.17856

Table 2: Percentage of misclassified dyads for four simulated relationship categories (PO: Parent-Offspring, FS: Full-Sibs, HS: Half-Sibs, UR: Unrelated) using different reference populations (entire – all samples pooled, Millstream, Ophthalmia Dam, Western Ridge), produced by the Wang (Wang 2002) estimator.

Error type	Simulated relationship	Misclassified as	Reference population			
			Entire	Millstream	Ophthalmia Dam	Western Ridge
Type I	UR	HS	24.4	30.2	28.2	32.6
Type II	HS	UR	22.4	25.2	22.4	26.6
Type I	HS	FS	24.8	30.2	23.4	28.2
Type II	FS	HS	19	22.6	20	20.2
Type I	HS	PO	25.4	30	23.4	28.2
Type II	PO	HS	10.8	15.2	10.6	13.6
Type I	UR	FS	1.2	5.8	2.2	6.4
Type II	FS	UR	0.8	2.8	2	2.4
Type I	UR	PO	1.4	5.8	2.2	6.4
Type II	PO	UR	0	0	0	0.2

Table 3: Reference population-specific threshold values used to distinguish dyads into relatedness categories (Parent-Offspring, Full-Sibs, Half-Sibs, Unrelated). Values to the left of the threshold are assigned to the higher relationship category, while values to the right of the threshold are assigned to the lower relatedness category.

	Parent-Offspring ⇔ Half-Sib	Full-Sib ⇔ Half-Sib	Half-Sib ⇔ Unrelated
Entire	0.376453	0.379984	0.1241097
Millstream	0.3775218	0.375669	0.1235675
Ophthalmia Dam	0.3752171	0.375528	0.1251317
Western Ridge	0.3688689	0.373129	0.1116207

Table 2: Pairwise relatedness values for all dyads with a relatedness value (*Rxy*) above the reference population threshold value for unrelated, and with confidence intervals that do not intersect 0.

95% confidence intervals are depicted in brackets. Threshold unrelated values: Entire = 0.1241097, Millstream = 0.1235675, Western Ridge = 0.111620, Ophthalmia Dam = 0.125131. Inferred relationship classes Parent-offspring (PO), Full-sibling (FS), and sampling sites Millstream (MS), Western Ridge (WR), Ophthalmia Dam (OD) are shown.

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
Entire	POP 101	POP 103	0.7305 (0.5003 - 0.898)	1st-degree (FS)	MS
	POP 106	POP 105	0.3929 (0.015 - 0.762)	1st-degree (FS)	MS
	POP 105	POP 104	0.5491 (0.3278 - 0.7567)	1st-degree (PO)	MS
	POP 202	QR17	0.484 (0.1564 - 0.7322)	1st-degree (PO)	WR
	QR17	POP 216	0.3886 (0.3056 - 0.5105)	1st-degree (PO)	WR
	QR17	QR41	0.6563 (0.4522 - 0.8663)	1st-degree (FS)	WR
	POP 204	POP 203	0.4605 (0.1392 - 0.724)	1st-degree (FS)	OD
	POP 203	POP 210	0.461 (0.1564 - 0.7365)	1st-degree (FS)	OD
	POP 204	POP 210	0.5225 (0.2391 - 0.7544)	1st-degree (PO/FS)	OD
	POP 210	POP 213	0.3205 (0.1548 - 0.487)	2nd-degree	OD
	POP 209	POP 214	0.5311 (0.3813 - 0.7365)	1st-degree (PO/FS)	OD
	POP 206	POP 222	0.3462 (0.2059 - 0.4406)	2nd-degree	OD
	POP 213	POP 215	0.4806 (0.3503 - 0.6705)	1st-degree (PO)	OD
	POP 215	POP 222	0.4787 (0.2851 - 0.6872)	1st-degree (PO)	OD
Millstream	POP 101	POP 103	0.6267 (0.306 - 0.8472)	1st-degree (FS)	MS
	POP 105	POP 104	0.3862 (0.1562 - 0.6123)	1st-degree (PO)	MS
Ophthalmia Dam	POP 204	POP 203	0.4321 (0.0494 - 0.7277)	1st-degree (FS)	OD
	POP 204	POP 210	0.4564 (0.1475 - 0.7187)	1st-degree (PO)	OD
	POP 203	POP 210	0.4203 (0.0981 - 0.7004)	1st-degree (FS)	OD
	POP 210	POP 213	0.2366 (0.051 - 0.4023)	2nd-degree	OD
	POP 209	POP 214	0.488 (0.3223 - 0.7)	1st-degree (PO)	OD
	POP 206	POP 222	0.3114 (0.1317 - 0.4207)	2nd-degree	OD
	POP 213	POP 215	0.4364 (0.2932 - 0.6387)	1st-degree (PO)	OD
	POP 213	POP 218	0.3707 (0.0481 - 0.6423)	2nd-degree	OD
	POP 214	QR44	0.4277 (0.0722 - 0.8132)	1st-degree (FS)	OD
	POP 215	POP 222	0.4583 (0.2091 - 0.6813)	1st-degree (PO)	OD

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
Western Ridge	QR17	POP 216	0.1944 (0.0077 - 0.3579)	2nd-degree	WR
	QR17	QR41	0.5494 (0.2498 - 0.8171)	1st-degree (PO)	WR

Table 3: Pairwise relatedness values for all dyads with a relatedness value (*Rxy*) above the reference population threshold value for unrelated, including those with confidence intervals that intersect 0.

95% confidence intervals are depicted in brackets. Threshold unrelated values: Entire = 0.1241097, Millstream = 0.1235675, Western Ridge = 0.111620, Ophthalmia Dam = 0.125131. Inferred relationship classes Parent-offspring (PO), Full-sibling (FS), and sampling sites Millstream (MS), Western Ridge (WR), Ophthalmia Dam (OD) are shown.

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
	POP 101	POP 103	0.7305 (0.5003 - 0.898)	1st-degree (FS)	MS
	POP 101	POP 105	0.1659 (-0.1671 - 0.4099)	2nd-degree	MS
	POP 101	POP 104	0.2075 (-0.1182 - 0.5676)	2nd-degree	MS
	POP 103	POP 102	0.2124 (-0.1485 - 0.5362)	2nd-degree	MS
	POP 103	POP 105	0.2471 (-0.1383 - 0.5255)	2nd-degree	MS
	POP 103	POP 104	0.1553 (-0.203 - 0.5164)	2nd-degree	MS
	POP 102	POP 105	0.1884 (-0.1384 - 0.4524)	2nd-degree	MS
	POP 102	POP 201	0.1609 (-0.4087 - 0.5733)	2nd-degree	MS/WR
	POP 106	POP 105	0.3929 (0.015 - 0.762)	1st-degree (FS)	MS
	POP 106	POP 211	0.1744 (-0.1835 - 0.4088)	2nd-degree	MS/WR
	POP 106	POP 203	0.2743 (-0.3043 - 0.6647)	2nd-degree	MS/OD
	POP 105	POP 104	0.5491 (0.3278 - 0.7567)	1st-degree (PO)	MS
	POP 105	POP 201	0.3162 (-0.0655 - 0.6486)	2nd-degree	MS/WR
Entire	POP 105	POP 212	0.212 (-0.1106 - 0.5575)	2nd-degree	MS/WR
	POP 105	QR41	0.2897 (-0.209 - 0.7153)	2nd-degree	MS/WR
	POP 104	POP 212	0.2116 (-0.1163 - 0.6022)	2nd-degree	MS/WR
	POP 201	POP 202	0.2434 (-0.1996 - 0.5938)	2nd-degree	WR
	POP 201	QR17	0.3269 (-0.0913 - 0.6763)	2nd-degree	WR
	POP 201	POP 212	0.2339 (-0.1946 - 0.6653)	2nd-degree	WR
	POP 201	QR41	0.3738 (0.0514 - 0.6437)	2nd-degree	WR
	POP 201	POP 217	0.2715 (-0.0992 - 0.5295)	2nd-degree	WR/OD
	POP 202	QR17	0.484 (0.1564 - 0.7322)	1st-degree (PO)	WR
	POP 202	POP 212	0.2794 (-0.0713 - 0.5887)	2nd-degree	WR
	POP 202	POP 216	0.3933 (0.1099 - 0.6311)	1st-degree (PO)	WR
	POP 202	QR41	0.3816 (0.1089 - 0.6179)	1st-degree (PO)	WR
	POP 202	POP 203	0.2306 (-0.2024 - 0.5669)	2nd-degree	WR/OD
	POP 202	POP 206	0.2092 (-0.2737 - 0.5176)	2nd-degree	WR/OD

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
	QR17	POP 212	0.2017 (-0.167 - 0.5181)	2nd-degree	WR
	QR17	POP 216	0.3886 (0.3056 - 0.5105)	1st- degree (PO)	WR
	QR17	QR41	0.6563 (0.4522 - 0.8663)	1st- degree (FS)	WR
	QR17	POP 206	0.1955 (-0.1506 - 0.4161)	2nd-degree	WR/OD
	POP 211	POP 216	0.1757 (-0.1544 - 0.4554)	2nd-degree	WR
	POP 212	QR41	0.1515 (-0.1805 - 0.5661)	2nd-degree	WR
	POP 212	POP 207	0.2334 (-0.1215 - 0.4705)	2nd-degree	WR/OD
	POP 216	QR41	0.3246 (0.0096 - 0.6965)	2nd-degree	WR
	QR41	POP 208	0.2082 (-0.1916 - 0.5384)	2nd-degree	WR/OD
	QR41	POP 206	0.1814 (-0.331 - 0.5329)	2nd-degree	WR/OD
	QR41	POP 222	0.1428 (-0.2675 - 0.4486)	2nd-degree	WR/OD
	POP 205	POP 203	0.1333 (-0.2473 - 0.5602)	2nd-degree	OD
	POP 205	POP 210	0.2845 (-0.1059 - 0.7292)	2nd-degree	OD
	POP 205	POP 207	0.3019 (-0.1097 - 0.7514)	2nd-degree	OD
	POP 204	POP 203	0.4605 (0.1392 - 0.724)	1st- degree (FS)	OD
	POP 204	POP 208	0.2017 (-0.1554 - 0.5188)	2nd-degree	OD
	POP 204	POP 210	0.5225 (0.2391 - 0.7544)	1st- degree (PO/FS)	OD
	POP 204	POP 213	0.1838 (-0.1797 - 0.4797)	2nd-degree	OD
	POP 204	POP 214	0.2421 (-0.1593 - 0.6524)	2nd-degree	OD
	POP 204	POP 218	0.163 (-0.3511 - 0.5425)	2nd-degree	OD
	POP 204	QR44	0.2385 (-0.1027 - 0.5796)	2nd-degree	OD
	POP 203	POP 210	0.461 (0.1564 - 0.7365)	1st- degree (FS)	OD
	POP 203	POP 207	0.3252 (0.0383 - 0.5552)	2nd-degree	OD
	POP 203	POP 222	0.2369 (-0.1696 - 0.5693)	2nd-degree	OD
	POP 203	QR45	0.2744 (-0.1997 - 0.7612)	2nd-degree	OD
	POP 208	POP 210	0.2405 (-0.1016 - 0.5884)	2nd-degree	OD
	POP 208	POP 206	0.1522 (-0.2837 - 0.5047)	2nd-degree	OD
	POP 208	POP 207	0.1536 (-0.203 - 0.6272)	2nd-degree	OD
	POP 208	POP 214	0.2641 (-0.0134 - 0.4476)	2nd-degree	OD
	POP 208	POP 222	0.277 (-0.0992 - 0.5678)	2nd-degree	OD

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
	POP 208	QR44	0.1851 (-0.199 - 0.4743)	2nd-degree	OD
	POP 210	POP 207	0.3103 (-0.0043 - 0.6548)	2nd-degree	OD
	POP 210	POP 213	0.3205 (0.1548 - 0.487)	2nd-degree	OD
	POP 210	POP 215	0.4162 (-0.0207 - 0.7289)	1st- degree (FS)	OD
	POP 210	POP 222	0.3632 (0.0129 - 0.63)	1st- degree (PO/FS)	OD
	POP 210	POP 218	0.2433 (-0.2297 - 0.5724)	2nd-degree	OD
	POP 210	QR45	0.252 (-0.0925 - 0.6059)	2nd-degree	OD
	POP 209	POP 213	0.2077 (-0.1743 - 0.5053)	2nd-degree	OD
	POP 209	POP 214	0.5311 (0.3813 - 0.7365)	1st- degree (PO/FS)	OD
	POP 209	POP 222	0.1445 (-0.2351 - 0.3948)	2nd-degree	OD
	POP 209	POP 217	0.138 (-0.2727 - 0.4294)	2nd-degree	OD
	POP 206	POP 214	0.189 (-0.2101 - 0.4804)	2nd-degree	OD
	POP 206	POP 222	0.3462 (0.2059 - 0.4406)	2nd-degree	OD
	POP 207	POP 213	0.1533 (-0.2029 - 0.4095)	2nd-degree	OD
	POP 213	POP 214	0.206 (-0.1581 - 0.5115)	2nd-degree	OD
	POP 213	POP 215	0.4806 (0.3503 - 0.6705)	1st- degree (PO)	OD
	POP 213	POP 218	0.4187 (0.1069 - 0.6808)	1st- degree (FS)	OD
	POP 213	QR44	0.2181 (-0.1199 - 0.4953)	2nd-degree	OD
	POP 214	POP 217	0.2832 (-0.0847 - 0.5526)	2nd-degree	OD
	POP 214	POP 218	0.1476 (-0.2191 - 0.3781)	2nd-degree	OD
	POP 214	QR44	0.4501 (0.099 - 0.819)	1st- degree (FS)	OD
	POP 215	POP 222	0.4787 (0.2851 - 0.6872)	1st- degree (PO)	OD
	POP 215	POP 218	0.3658 (0.0183 - 0.6225)	2nd-degree	OD
	POP 218	QR44	0.2549 (-0.1114 - 0.5283)	2nd-degree	OD
Millstream	POP 101	POP 103	0.6267 (0.306 - 0.8472)	1st- degree (FS)	MS
	POP 106	POP 105	0.1798 (-0.1924 - 0.6271)	2nd-degree	MS
	POP 105	POP 104	0.3862 (0.1562 - 0.6123)	1st- degree (PO)	MS
Ophthalmia Dam	POP 205	POP 210	0.2186 (-0.1859 - 0.6989)	2nd-degree	OD
	POP 205	POP 207	0.2452 (-0.1837 - 0.7453)	2nd-degree	OD
	POP 204	POP 203	0.4321 (0.0494 - 0.7277)	1st- degree (FS)	OD

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
	POP 204	POP 208	0.148 (-0.241 - 0.4807)	2nd-degree	OD
	POP 204	POP 210	0.4564 (0.1475 - 0.7187)	1st- degree (PO)	OD
	POP 204	POP 214	0.1527 (-0.2631 - 0.6296)	2nd-degree	OD
	POP 204	QR44	0.1609 (-0.1913 - 0.5471)	2nd-degree	OD
	POP 203	POP 210	0.4203 (0.0981 - 0.7004)	1st- degree (FS)	OD
	POP 203	POP 207	0.2827 (-0.0364 - 0.5288)	2nd-degree	OD
	POP 203	POP 222	0.175 (-0.2548 - 0.515)	2nd-degree	OD
	POP 203	QR45	0.1714 (-0.3139 - 0.7147)	2nd-degree	OD
	POP 208	POP 210	0.1977 (-0.1927 - 0.5567)	2nd-degree	OD
	POP 208	POP 207	0.1527 (-0.2923 - 0.6489)	2nd-degree	OD
	POP 208	POP 214	0.1901 (-0.1028 - 0.3998)	2nd-degree	OD
	POP 208	POP 222	0.2257 (-0.1971 - 0.5339)	2nd-degree	OD
	POP 210	POP 207	0.2654 (-0.0646 - 0.6203)	2nd-degree	OD
	POP 210	POP 213	0.2366 (0.051 - 0.4023)	2nd-degree	OD
	POP 210	POP 218	0.1605 (-0.3606 - 0.5246)	2nd-degree	OD
	POP 210	QR45	0.1904 (-0.1829 - 0.5616)	2nd-degree	OD
	POP 209	POP 214	0.488 (0.3223 - 0.7)	1st- degree (PO)	OD
	POP 206	POP 222	0.3114 (0.1317 - 0.4207)	2nd-degree	OD
	POP 213	POP 215	0.4364 (0.2932 - 0.6387)	1st- degree (PO)	OD
	POP 213	POP 218	0.3707 (0.0481 - 0.6423)	2nd-degree	OD
	POP 213	QR44	0.1526 (-0.239 - 0.4453)	2nd-degree	OD
	POP 214	POP 217	0.216 (-0.1965 - 0.5316)	2nd-degree	OD
	POP 214	QR44	0.4277 (0.0722 - 0.8132)	1st- degree (FS)	OD
	POP 215	POP 222	0.4583 (0.2091 - 0.6813)	1st- degree (PO)	OD
	POP 215	POP 218	0.3276 (-0.031 - 0.5986)	2nd-degree	OD
	POP 218	QR44	0.2041 (-0.2373 - 0.5265)	2nd-degree	OD
Western Ridge	POP 202	QR17	0.2254	2nd-degree	WR
	POP 202	POP 216	0.1812	2nd-degree	WR
	POP 202	QR41	0.1226	2nd-degree	WR
	QR17	POP 216	0.1944	2nd-degree	WR

Reference population	Individual 1	Individual 2	*Rxy*	Inferred relationship	Sample site
	QR17	POP 216	0.1944	2nd-degree	WR