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Evidence of mitochondrial capture in Australian glass shrimp (*Paratya australiensis*) in south-eastern Queensland

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ABSTRACT

Context. The Australian glass shrimp (*Paratya australiensis*) has been identified as a cryptic species complex and several lineages occur sympatrically in eastern Australia. In south-eastern Queensland, the predominant lineages are '4' and '6', although the only known area of sympatry in the Conondale Range is the result of a translocation. **Aim.** Our aim was to determine any evidence of natural sympatry between the two lineages in the Mary River catchment area using polymerase chain reatction–restriction fragment length polymorphism (PCR-RFLP) analysis. **Methods.** Of six sites sampled, only one site showed sympatry between Lineage 4 and 6, so 39 individuals from this site were sequenced using fragment of the mtDNA *COI* gene and a neighbour joining tree revealed the existence of two lineages. **Results.** On the basis of two sets of nuclear markers (allozymes and microsatellites), there was no evidence of two species, because all loci conformed to Hardy–Weinberg expectations and only Lineage 4 alleles were identified. **Conclusion.** These results led to the inference that the existence of two mtDNA lineages could be due to mitochondrial capture. **Implication.** The current situation in the Broken Bridge High site could be due to historical interbreeding between the two lineages reflected in the mtDNA data but not evident in the nuclear data.

Keywords: cryptic species, freshwater shrimp, historical interbreeding, hybridisation, lineages, mitochondrial DNA, nuclear DNA, sympatry.

Introduction

When two or more species are superficially morphologically indistinguishable and considered as one single species, they are known as cryptic species (Bickford *et al.* 2007). The cryptic species concept was recognised 300 years ago, but with the advancement of DNA sequencing techniques, research on cryptic species identification has become more convenient and popular (Leys *et al.* 2016). Tropical rainforests have been considered as hotspots for cryptic species because they are species-rich habitats with many different microhabitats. Fungi, frogs and arthropods are well known for having cryptic-species complexes (Bickford *et al.* 2007). The different species of arthropods have been important in studies of coevolution and species interactions (Bickford *et al.* 2007). Furthermore, cryptic species may show varying levels of adaptive divergence and reproductive isolation (Bickford *et al.* 2007). In many cases, cryptic species exist in sympatry, yet remain reproductively isolated (Cook *et al.* 2007). Such sympatric cryptic species are distributed widely in various ecosystems (Pfenninger and Schwenk 2007). They may possess unique adaptations and evolutionary potential; hence, they should be considered as separate evolutionary units when studying their ecology and evolution as well as developing conservation plans (Crandall *et al.* 2000).

Cryptic species may arise either allopatrically or sympatrically, but in many cases they now co-occur at the same site and are reproductively isolated. Cook *et al.* (2007) reported that two lineages of *Paratya australiensis* in the Granite Creeks area in south-eastern Australia co-occurred but were reproductively isolated.

If lineages of cryptic species co-occur, there are four possible scenarios.

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Scenario A: there might be free mixing or interbreeding between the lineages

If two lineages are interbreeding and there is symmetrical hybridisation, the population should conform to predictions of Hardy–Weinberg equilibrium (HWE) for each nuclear locus observed, because there will be free mating between lineages (Allendorf *et al.* 2012). Again, there could be deep mitochondrial lineages that resulted from past allopatry that allowed for genetic drift in mitochondrial, but not in the nuclear, genome and even if there was nuclear divergence, it was not enough to stop breeding and subsequent reblending when the mitochondrial lineages came back together again. This scenario is that they are, and always have been, one species (Dasmahapatra *et al.* 2010).

Scenario B: the two lineages may not interbreed or mix at all

If the two lineages maintain separate genepools and do not interbreed at all, the population should deviate significantly from HWE expectations and there will be a deficit of heterozygotes compared with expectations (Cook *et al.* 2007). Some loci may be fixed for alternate alleles; also, if nuclear genomes have differentiated, then it would likely show significant linkage disequilibrium. This scenario is that they are distinct, biological species.

Scenario C: there could be asymmetrical hybridisation

In this case there could be contemporary hybridisation between two lineages but introgression may not occur between the two lineages due to sterile hybrids or less fit hybrids. Fawcett *et al.* (2010) observed such asymmetrical hybridisation at the translocation site (two subcatchments of Brisbane River) where a translocated lineage was sending the resident lineage to extinction because of non-viable hybrids between resident females and translocated males.

Scenario D: there could have been historical interbreeding which may have led to mitochondrial capture

In this case, mtDNA would show presence of two lineages as hybridisation between the lineages may have taken place historically leading to mitochondrial introgression from one lineage to the other. If mitochondrial capture has occurred, the population would appear as one single lineage on the basis of nuclear data, but as two lineages on the basis of mtDNA, owing to past mitochondrial introgression (Good *et al.* 2008). With historical mitochondrial introgression, if the invader population became larger than the resident population or if there were sex-biased reproduction during hybridisation, then there would be replacement of the mitogenome of one species with that of the other. As a result, eventually one nuclear lineage will go extinct (Perea *et al.* 2016). This scenario is that only one species is present, but some of them can carry the mitochondrial DNA of another species.

The glass shrimp, *P. australiensis*, is a freshwater taxon that is highly abundant in the subtropical rainforest streams of south-eastern Queensland, Australia. It has been reported to consist of a number of cryptic species. Nine highly divergent mtDNA lineages have been identified so far (Cook *et al.* 2006). Lineages 3, 5 and 7 are restricted to single rivers, Lineages 1, 2 and 9 are restricted to geographically proximate rivers and Lineages 4, 6 and 8 have been shown to be widespread throughout eastern Australia, occurring in both coastal streams and inland rivers flowing into the Murray–Darling system (Cook *et al.* 2006).

These lineages of *P. australiensis* have been found together in various combinations (Cook *et al.* 2006). In some instances where they co-exist, they have been shown to be reproductively isolated (Cook *et al.* 2007) as in Scenario B. For example, in one creek in southern Australia (Granite Creek), Lineages 4 and 8 co-occur, but do not interbreed (Cook *et al.* 2007). In south-eastern Queensland, Lineages 3, 4 and 8 have been recorded to co-occur in streams on the Gold Coast, Queensland (Garzon 2009).

So as to study the movement patterns of *P. australiensis*, a translocation event took place in 1993 between two creeks in the Brisbane River Catchment in south-eastern Queensland. At the time, it was not known that the shrimps in the two streams belonged to different lineages. However, it was known that there were fixed differences at three allozyme loci. These loci were then used as markers to observe subsequent movement up and down the stream (Hancock and Hughes 1999). It was only later, when mtDNA was sequenced from each of the populations (Hurwood *et al.* 2003), that it became evident that two genetically divergent lineages had been mixed, later named Lineage 4 and Lineage 6 (Cook *et al.* 2006). Further monitoring of the streams showed that in one stream (Kilcoy Creek), the lineage from the translocated population quickly disappeared, whereas in the other (Branch Creek), the translocated lineage took over, both above and below the original translocation site (Hughes *et al.* 2003; Wilson *et al.* 2016). The translocated 'mtDNA lineage' replaced the resident lineage in Branch Creek through non-random mating. Subsequently, Rogl (2021) undertook genome-wide single-nucleotide polymorphism (SNP) analysis in the Branch Creek hybrid zone. She showed that, in many instances, shrimp with the introduced Kilcoy mtDNA lineage had nuclear genomes more similar to those in Branch Creek. Further, genetic analysis has demonstrated that limited interbreeding has occurred between the two lineages, although this seems to have been more extensive soon after the translocation than it is now (Fawcett *et al.* 2010; Wilson *et al.* 2016). In fact, recently, Lineage 4 has moved further downstream and appears to

have displaced Lineage 6 with only two sites where both still occur (Wilson *et al.* 2016). In 2007 and 2008, Garzon sampled a number of creeks to determine where Lineages 4 and 6 co-occurred naturally (Garzon 2009). At that time, he was unable to find Lineage 4 and Lineage 6 together, despite finding a number of the other lineages co-occurring, often in transition zones between upstream and downstream portions of creeks. Eight sites on the Mary River were sampled by Hurwood *et al.* (2003). All contained only Lineage 4.

Examination of co-occurring lineages in *P. australiensis* can facilitate examination of hybridisation in nature. It was suggested in previous studies in the translocation area (artificial) that there was asymmetrical hybridisation between Lineage 4 and Lineage 6 (Hughes *et al.* 2003; Garzon 2009; Fawcett *et al.* 2010; Wilson *et al.* 2016). This was because; F1 hybrids all had Lineage 4 mtDNA, which would have resulted from mating between Lineage 4 females and Lineage 6 males. In relation to these events, we sampled six sites in the Mary River for a project related to adaptation to altitude in *P. australiensis* and we had to identify the lineage of each of the samples (as we were interested in only Lineage 4). So, this was a perfect opportunity to observe more sites to determine whether both Lineage 4 and Lineage 6 co-occurred naturally, or whether, as suggested by the work of Wilson *et al.* (2016), one tended to displace the other, possibly depending on the elevation of the site. Lineage 4 tends to occur at higher elevations than does Lineage 6 and has been shown to be less tolerant of high temperature (Fawcett *et al.* 2010), so we expected that co-occurrence, if it was observed, would be at intermediate elevations.

Here we sampled six sites in three different tributaries of the Mary River and used mtDNA sequencing combined with polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) tests to determine which lineage occurred in each site. Furthermore, we used allozyme and microsatellite markers to observe whether or not there was interbreeding if more than one lineage occurred at a site. Given the results of the translocation experiment, we predicted that if the two lineages did co-occur, they would be at an intermediate altitude and that one lineage would be common and the other rare.

Materials and methods

Sampling and DNA extraction

Six sites had been sampled in the Conondale Range (26°55′S, 152°45′E, north-west to 26°37′S, 152°30′E) north-west of Brisbane (Fig. 1). High-elevation sites were above ~550 m above sea level (ASL). and low-elevation sites were ~100 m ASL. These data were obtained using a GPS. High and low sites were ~50 km apart from each other. From the six sites, 100–150 specimens were collected from each site.

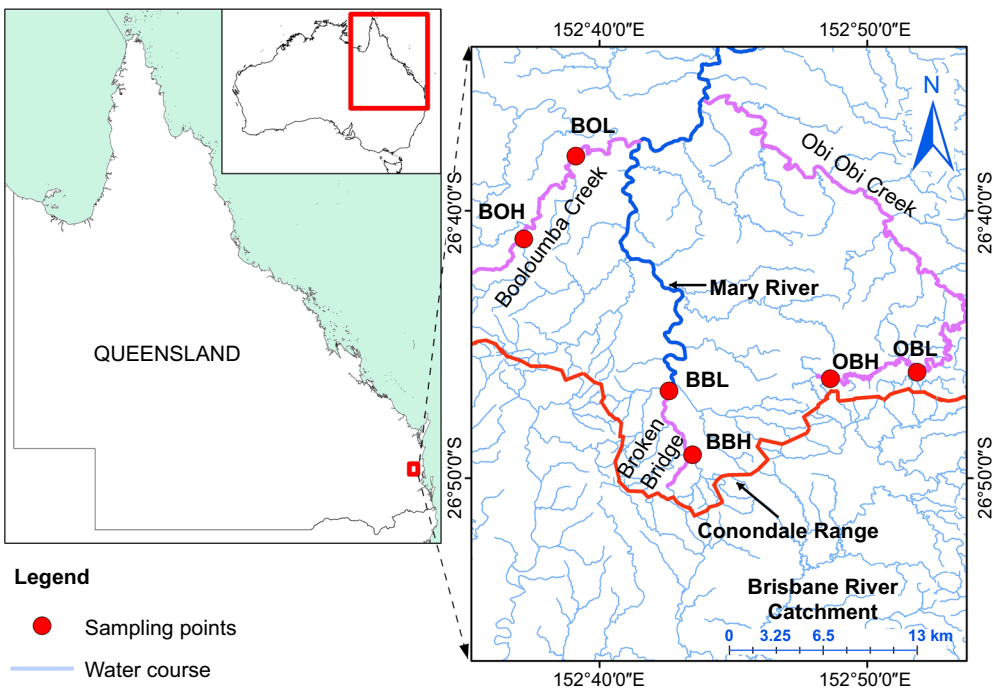

Fig. 1. Map of the Conondale Range showing sampling sites. BOH, Booloumba Creek High; BOL, Booloumba Creek Low; BBH, Broken Bridge High; BBL, Broken Bridge Low; OBH, Obi Obi High; OBL, Obi Obi Low. Sampling sites are indicated with red circles in the map.

P. australiensis individuals were captured using a seine net. Shrimps were immediately preserved in 100% ethanol. DNA was extracted from all specimens collected to yield high-quality genomic DNA using DN-easy Blood and Tissue kit (QIAGEN, GmbH, D-40724, Hilden, Germany), following the manufacturer's instructions. In total, 270 individuals had the best high-quality genomic DNA (required for another project) and 270 samples from the six sites were identified as Lineages 4 or 6 by using PCR-RFLP. At the Broken Bridge High site, where more than one lineage was detected, 120 individuals were tested for PCR-RFLP. So, among these 120 individuals, 39 individuals were sequenced using a fragment of the cytochrome *c* oxidase subunit I (*COI*) gene to confirm species identification.

PCR-RFLP

The solution used to amplify *COI* fragment contained 5× Mytaq buffer, 10 μM of Para 4/6 forward primer (Wright 2012), 10 μM of para 4/6 reverse primer (Wright 2012), 5 U μL^{-1} of Mytaq, 100 ng of DNA. The program used on the thermocycler was as follows: 5 min at 94°C for denaturation, 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C and a last extension cycle at 72°C for 7 min.

The restriction enzymes were Pho-I and Tse-I, as was used by Garzon (2009). *Pho*-I cuts the *COI* sequence of individuals of Lineage 4 into two fragments (300–150 bp) but does not cut *COI* sequence of individuals from Lineage 6. By contrast, *Tse*-I cuts the *COI* sequence of individuals from Lineage 6 into two fragments (300–150 bp), but does not cut the segment from Lineage 4 individuals.

PCR product was digested using 6.3 μL of ddH_2O, 1.0 μL of Cut Smart Buffer, 50 U mL^{-1} of Pho-I/Tse enzyme, 5 μL of PCR DNA, and incubated at 75°C for 1 h for Pho-I and at 65°C for 2 h for Tse-I. Lineages were distinguished on 0.8% agarose gel electrophoresis.

Barcoding

DNA extractions were used for mtDNA PCR of the *COI* fragment by using standard LCO1490 and HCO2198 primers (Folmer *et al.* 1994). The PCR protocol used started with an initial cycle of denaturation at 94°C for 5 min and was followed by 15 cycles of 30 s at 94°C, 30 s at 45°C, 30 s at 72°C and another 25 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C and a last extension cycle at 72°C for 7 min. The success of the PCR was checked on a 0.8% agarose gel at 80 V for 40 min. PCR product was cleaned prior to sequencing with exoSAP (Promega), following Werle *et al.* (1994). In total, 39 individuals of the 120 individuals from the Broken Bridge High (BBH) Site were sequenced using Macrogen Inc. sequencing facilities (Seoul, South Korea). PCR products were sequenced in the forward direction by using LCO1490 primer only.

Phylogenetic analysis

Sequences were aligned and edited using SEQUENCHER (ver. 4.1, Gene Codes Corporation, see http://www.genecodes.com/). Sequences were submitted to GenBank and the accession numbers ranged from MK312168 to MK312206 for 39 individuals (Supplementary Table S1). Haplotype diversity, number of haplotypes, number of monomorphic and polymorphic sites of the mtDNA sequences (for 39 individuals) was calculated using DNASP (ver. 6, Universitat de Barcelona, Spain, see http://www.ub.es/dnasp; Rozas *et al.* 2017). Besides, nucleotide diversity (Nei 1987) and haplotype diversity of each group (Clade A and Clade B based on mtDNA data) were calculated in Arlequin (ver. 3.5.1.2, see http://cmpg.unibe.ch/software/arlequin35; Excoffier and Lischer 2010).

Because the predominant lineages in Queensland are Lineages 4 and 6, mtDNA sequences already published were used as references to align the new samples. The reference sequences used were GenBank Accession numbers AF534894–AF534904 (Hurwood *et al.* 2003). Also, a sequence of *Paratya howensis* (GenBank Accession number AY622605; Lord Howe Island) was used as an outgroup (Page *et al.* 2005). A Neighbour-joining (NJ) tree was produced using the HKY distance model in Geneious (ver. 9.1.5, see https://www.geneious.com/; Kearse *et al.* 2012), with 1000 bootstrap replicates.

Microsatellite procedure

Sequencing, primer design and initial locus testing

Sequencing of microsatellite loci was undertaken according to Wilson *et al.* (2016). Three loci showed distinct genetic differences between the two lineages (Wilson *et al.* 2016) and these three loci, namely, Ion 09, Ion 44 and 454-36 were used for the present study (Table S2). In total, 24 individuals from BBH site were amplified for these three loci.

Single loci were amplified using PCR. The PCR mix contained 1× reaction buffer, 1.5 mM of $MgCl_2$, 0.1 μM of forward primer and 0.4 μM of reverse primer, 0.4 μM of fluorescent tag (Real *et al.* 2009), 1% of bovine serum albumin, 0.2 mM of deoxynucleotide triphosphates (dNTPs), 50 U mL^{-1} of white *Taq* polymerase and 100 ng of DNA. Thermocycler conditions were as follows: 94°C for 5 min (initial denaturing), 35 cycles of 94°C for 30 s (denaturing), 55°C for 30 s (annealing) and 72°C for 7 min (extension) the solution was then held at 72°C for 7 min (final extension).

Fragment analysis of microsatellites was conducted on an Applied Biosystems Genetic Analyser 3130. Microsatellites were scored using Genemapper (ver. 3.1, Applied Biosystems).

Allozyme markers

In addition to the mtDNA and microsatellite markers, three allozyme markers were also used for this study. These were

chosen because they had previously been shown to differ significantly between the two lineages (Hurwood *et al.* 2003).

The allozyme loci are AAT-1, AAT-2 (IECC number = 2.6.1.1) and MPI (IECC number 5.3.1.8) (Hughes *et al.* 1995). Cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, TX, USA) were used for electrophoresis. AAT-1 and AAT-2 were run for 60 min in Tris glycine (pH 8.5) and MPI was run on Tris-citrate (pH 7.0) for 50 min before staining. Stains were mixed with 1.5% agar (~2 mL) and applied to the plates. Plates were left for 10 min until the agar had set, then scored. In total, 36 individuals from BBH site were analysed at these three loci.

For the initial project, DNA was extracted from many individuals (600–900 individuals) and we selected only the best ones with very high-quality DNA for RFLP test to check whether they were Lineage 4/6. So, the 270 individuals had the best-quality DNA for RAD-seq. From six sites, these individuals were subjected to RFLP test with Pho and Tse restriction enzymes to determine which lineage (Lineage 4/6) they belonged to. We were interested in Lineage 4 for the other project, but we identified Lineage 6 in one site. So, among these 270 individuals, 120 individuals from BBH site had presence of both lineages (4 and 6). So, we randomly selected 39 individuals from 120 individuals and sent them to Macrogen for sequencing (mtDNA *COI* gene).

When we found evidence with mtDNA that there were two lineages (Lineages 4 and 6) within 39 individuals at BBH, we decided to do more sampling at this site for studying the nuclear genome. On this occasion we analysed microsatellite markers on 24 individuals and allozyme markers on 36 individuals.

HWE test of microsatellite and allozyme data

HWE tests were undertaken with the exact test for allozyme and microsatellite loci using Arlequin (ver. 3.5.1.2; Excoffier and Lischer 2010).

Results

Co-occurring lineages and phylogenetics

The RFLP results from all sites from 270 individuals showed that of the six sites, only BBH (BBH in Fig. 1) contained more than one lineage. In total, 67% of the individuals were Lineage 6 and 33% of the individuals were Lineage 4. For the remaining five sites, all individuals belonged to Lineage 4, with no evidence of Lineage 6 (Table S3). So, on the basis of the RFLP results, for the first time, there was evidence of lineages of *P. australiensis* Lineages 4 and 6 occurring sympatrically.

Because the RFLP results showed that only one site contained both lineages, samples from only that site were sequenced. Direct sequencing of the *COI* mtDNA gene yielded 710 bp of sequence for 39 individuals from BBH and, after editing, a 614-bp sequence was used for analysis. Sequences were aligned with reference sequences. Six haplotypes were identified (Table S4). All the reference haplotypes were different by a single nucleotide site. Haplotype diversity was H_d = 0.7193. In all, 572 sites were monomorphic and 42 sites were polymorphic. The neighbour-joining tree showed two well supported clades (Fig. 2). Nineteen individuals from Broken Bridge Creek grouped in one clade with reference individuals from Stony Creek (Lineage 6: AF534902, AF534903 and AF534904; Fig. 2, Clade A) in the Brisbane River (Hurwood *et al.* 2003). In the other clade, 20 individuals from Broken Bridge Creek grouped together with reference samples from Hurwood *et al.* (2003) (Lineage 4, Brisbane River: AF534895, AF53899, AF534898, AF534897 and AF534900). Some of the reference samples were from the same site in the Mary River (AF534901, AF534894, AF534896 and AF534895; Fig. 2; Clade B). This grouping in the phylogenetic tree further supports coexistence of two lineages in the Mary River catchment area.

Nucleotide diversity within the two major groups (Clade A and Clade B, excluding reference samples) was 0.00120 ± 0.001045 (averaged over loci) for Clade A and 0.00143 ± 0.00169 for Clade B. Haplotype diversity for Clade A was H_d = 0.307 (averaged over four polymorphic nucleotide sites) and for Clade B H_d = 0.542 (averaged over five polymorphic nucleotide sites).

Investigating interbreeding with nuclear markers

The numbers of individuals used for microsatellite and allozyme analysis were different because not all individuals worked for the microsatellite markers. We omitted the ones with too much missing data.

For the three allozyme loci, 1 locus (AAT-2) was monomorphic and the other two (AAT-1 and MPI) were polymorphic. No alleles were identified that had previously been recorded as belonging to Lineage 6 (Fawcett *et al.* 2010) on the basis of these three loci and all 36 individuals showed alleles previously recorded for Lineage 4. The AAT-1 locus had two alleles and the MPI locus had three alleles (Table S5). For the microsatellite data, each locus was polymorphic, with two alleles for Ion 9, three alleles for Ion 44 and four alleles for 454_36, and when comparing these alleles with pure Lineage 4 and Lineage 6 alleles from Wilson *et al.* (2016), we did not find any pure Lineage 6 individuals in our data. (Table S5).

The HWE tests on the microsatellite and allozyme loci at the BBH site showed that genotype proportions conformed to Hardy–Weinberg predictions (Table S6). This result indicated that on the basis of these loci, individuals were randomly mating.

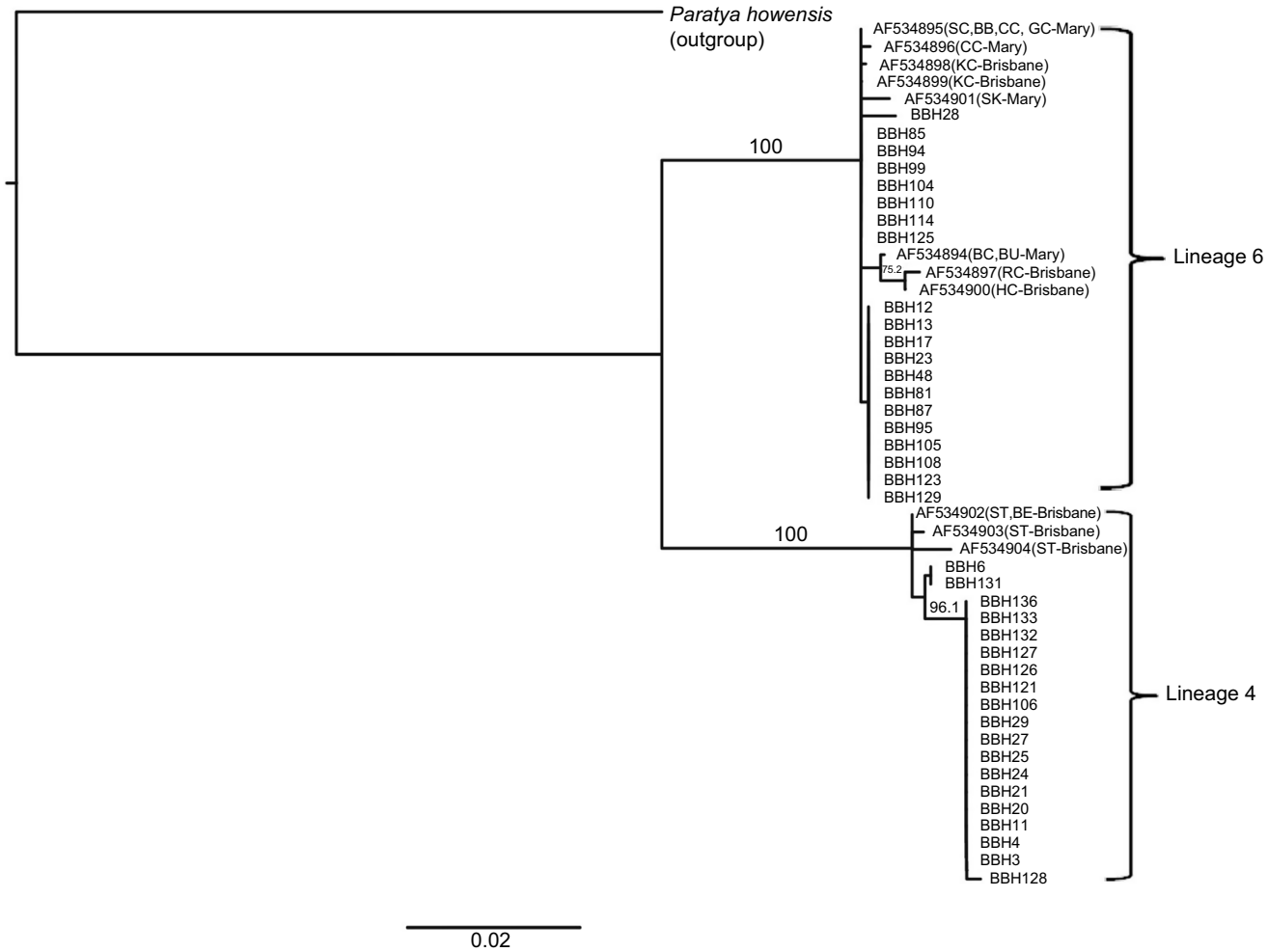

Fig. 2. *COI* mtDNA gene tree for *Paratya australiensis* generated using Geneious (ver. 9.1.5) including 39 samples, 11 reference sequence (Hurwood *et al.* 2003) and 1 outgroup *Paratya howensis*. Bootstrap values are indicated on the branches. Tips represent individuals of *P. australiensis*. Clade A includes lineage 6 individuals and clade B includes lineage 4.

Discussion

The present study aimed to identify any evidence of natural sympatry between Lineages 4 and 6 in the Mary River and to also test the hypothesis that if the lineages did co-occur, this would be at an intermediate altitude. Of the six study sites in the Mary River catchment, only one site had two lineages co-occurring on the basis of the mtDNA data. The neighbour-joining tree shows two lineages in two separate clades that grouped with the reference samples from Lineages 4 and 6 (Hurwood *et al.* 2003) respectively. The existence of sympatric lineages in this study aligns with one of Avise's phylogeographical categories (Category II), where major lineages identified in a gene tree are sympatric and sympatry is the result of admixture of previously allopatric populations (Avise 2000).

We predicted that the lineages would co-occur at an intermediate elevation (such as ~250 m ASL), but the area of sympatry was detected at an elevation of >~500 m. It was suggested by Cook *et al.* (2006) that Lineage 4 prefers to reside at higher elevations than does Lineage 6, which has mostly been recorded from lower elevations. In the current study, Lineages 4 and 6 were observed in a 1:2 ratio (on the basis of the RFLP result), similar to findings at the translocation site in the Brisbane River Catchment (Wilson *et al.* 2016). It has been mentioned by Leys *et al.* (2016) that the proportion of lineages at one site is likely to be influenced by environmental factors such as elevation and stream type. Although Lineage 6 has been suggested to prefer low altitudes in previous studies (Cook *et al.* 2006; Fawcett *et al.* 2010; Wilson *et al.* 2016), in our study Lineage 6, on the basis of mtDNA data, did not seem to be restricted by high elevation. Influence of elevation on *Paratya* population was reported by Cook *et al.* (2006) who mentioned that *Paratya* populations were isolated by steep stream gradients among the upland sites and between

upland and lowland sites in Granite Creek in Victoria. Also, Rahman *et al.* (2020) found highest genetic differentiation between high-elevation and low-elevation populations (*Paratya*) in Booloumba Creek in Queensland. So, there is evidence for influence of elevation on *Paratya* populations.

It was also observed that some individuals from the Mary River catchment grouped together with the reference samples (AF534901, AF534894, AF534895 and AF534896) from the Mary River (Clade B). This could be because reference samples and studied samples were collected from the same or a nearby site. Reference lineage 6 samples are slightly different from the studied samples according to the phylogenetic tree (Clade A). Lineage 6 samples (Hurwood *et al.* 2003) were recorded from Branch Creek East, Branch Creek West and Stony Creek in the Brisbane River, whereas the studied samples were recorded in Broken Bridge Creek in the Mary River. Hurwood *et al.* (2003) noticed sharing of haplotypes between Brisbane and Mary River catchments and suggested that contemporary dispersal may be responsible for the scenario. Again, genetic diversity was higher in Lineage 4 than in Lineage 6, which is also evident in previous results of Cook *et al.* (2006) who observed that Lineage 4 consists of five sublineages and is widely distributed across eastern Australia, whereas Lineage 6 has two sublineages and is not so widely distributed.

On the basis of the nuclear data (allozyme and microsatellite data), there was only one lineage and it was randomly mating according to the HWE results. There was no sign of heterozygote deficiency for any nuclear loci on the basis of microsatellite and allozyme data. So, in the current situation, we infer that there has been historical interbreeding between Lineages 4 and 6, which has led to mitochondrial capture. Evidence of mitochondrial capture has been reported in several studies, for example, in crustacean (*Daphnia pulex*) complex (Markova *et al.* 2013), in pine trees (*Pinus*; Wang and Wang 2014), in North American collared lemmings (*Dicrostonyx groenlandicus* and *Dicrostonyx hudsonius*; Fedorov *et al.* 2022), in chipmunks (*Tamias ruficaudus* and *Tamias amoenus*; Good *et al.* 2008) and in birds (Australasian monarch-flycatcher, *Symposiachrus trivirgatus;* and spotted winged monarch, *Symposiachrus guttula*; Andersen *et al.* 2021). In all these studies, there was discordance between nuclear and mitochondrial DNA data, leading to the interpretation that mitochondrial capture took place.

On the basis of the allozyme data, there were no alleles for Lineage 6 and microsatellite data had a mixture of alleles from Lineage 4 and Lineage 6, with Lineage 4 alleles being dominant. So, there is no strong evidence for the existence of Lineage 6 in this site, leading to the assumption that there is actually only one lineage, Lineage 4.

Initially, it appears as though the allozyme data and the microstatellite data are suggesting different scenarios. The allozyme data suggest all alleles present in BBH are those of Lineage 4, whereas the microsatellite data suggest that the BBH population contains a mixture of Lineages 4 and 6. These conflicting results could be explained in two ways. First, the BBH population could be a mixture of both lineages, including hybrids, but the Lineage 6 allozyme alleles have been lost, possibly through selection, whereas the neutral microsatellite genes of both lineages have been retained. Second, the sites chosen as controls for each lineage by Wilson *et al.* (2016) may not have been truly representative of each lineage. For example, although the controls appear to be monomorphic for alternative alleles, it is quite likely that broader sampling may have indicated wider variation. If this is the case, then the most likely scenario is that there has been mixing of the two lineages in the past, explaining multiple mtDNA lineages, but that only Lineage 4 alleles have survived.

It is possible, given that Lineage 6 usually prefers lower altitudes (Fawcett *et al.* 2010; Wilson *et al.* 2016), that Lineage 6 alleles were not well adapted to the conditions at the site and did not persist for long after the initial hybridisation. However, when we sampled further downstream in this Creek (Broken Bridge), we did not find any Lineage 6 individuals. Even when the two lineages were together in the artificially sympatric condition, hybrids from Lineage 6 females did not appear to survive, because none was recorded. It was proposed earlier that at the translocation-site Lineage 6 females were breeding with Lineage 4, but the offspring did not appear to survive, as none was recorded (Hughes *et al.* 2003; Fawcett *et al.* 2010). However, this proposal was not confirmed experimentally. In the current study, we do not think this condition applies, because we identified only one lineage here, Lineage 4, on the basis of nuclear data, and there is no hybridisation.

In conclusion, it can be said that mitochondrial gene tree provides strong phylogenetic estimates (Page *et al.* 2007); however, sole dependence on mitochondrial DNA data can lead to misinterpretation where mitochondrial capture prevails.

Supplementary material

Supplementary material is available online.

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Data availability. Sequences that were generated for this paper were deposited in the GenBank and the Accession numbers of the sequences are in Supplementary table (Table S1).

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