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Leech breach: a first record of the invasive freshwater leech *Helobdella europaea* (Hirudinea: Glossiphoniidae) in Fiji

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ABSTRACT

Context. The freshwater flat leech *Helobdella europaea* Kutschera, 1987 is a small annelid indigenous to South America. This invasive species feeds on the haemolymph of host aquatic invertebrates, with occurrences reported from Europe, USA, Taiwan, North Africa, Hawai'i, Australia and New Zealand. A large number of individuals were discovered in the Ba River catchment, Fiji, during a 2015–2020 freshwater biodiversity survey, raising concerns of potential impacts on endemic Fijian aquatic invertebrate fauna and ecosystem integrity. **Aims.** To facilitate assessments of its spread and ethology, this study employed morphological and phylogenetic analyses for verification of taxonomic identity. **Methods.** Phylogenetic trees were constructed using a 658 bp fragment of the mitochondrial DNA *cox1* (*COI*) gene. The first complete mitochondrial genome sequence of *H. europaea* was also determined using selective multiple displacement amplification and Oxford Nanopore Technology to provide a reference for future comparative analyses and source tracking of spread to other regions. **Key results.** Morphological and *COI* analyses identified all Fijian leech specimens collected ($n = 16$) as *H. europaea*, reporting the first occurrence of this species on a south-west Pacific Island. The complete mitochondrial genome was sequenced. **Conclusions.** Confirmation of its presence in Fiji is a national biosecurity concern and will guide the Biosecurity Authority of Fiji and national agencies in further ecosystem assessment and response strategies. **Implications.** With the complete mitochondrial genome of *H. europaea* now available, transmission pathway traceability is possible in other regions where this species may be detected.

Keywords: biosecurity, DNA barcoding, freshwater, invasive, leech, mitochondrial DNA, taxonomic identification, traceability.

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Introduction

Globally over 50% of the 917 described species of leeches (Mãgalhaes *et al.* 2021) inhabit freshwater and are divided among 91 genera (Sket and Trontelj 2008). Some freshwater leeches are highly specialised and play important roles as invertebrate predators. The family Glossiphoniidae is the sole lineage within the order Rhynchobdellida (proboscis-bearing leeches) that is predominantly confined to freshwater, although some taxa display temporary salinity tolerance (Sawyer 1974). Of all freshwater leeches, the genus *Helobdella* (Blanchard, 1896) (Annelida: Clitellata: Rhynchobdellida: Glossiphoniidae) is the most speciose, with 35 described species (Sket and Trontelj 2008). This genus has the highest diversity in South America with 20 species described (Sawyer 1986) and has received the most attention in the fields of annelid developmental biology, ecology, ethology and population genetic studies (Weisblat and Huang 2001; Seaver 2003).

Over the last 40 years the invasive freshwater flat leech, *Helobdella europaea* Kutschera 1987 has recorded occurrences in locations extending from the Palaearctic to the Australasian and Oceanian realms. *Helobdella europaea* belongs to the *Helobdella triserialis* species complex, members of which are indigenous to South America (Sawyer 1986). This

species is relatively small (1–8 cm long), two eyed, flat, with a generally grey coloured body and light pigment spots. It is hermaphroditic, feeds on the haemolymph of host aquatic invertebrates (oligochaetes, insects and gastropods), and conducts brood care of its young. While its ecological niche varies from still waters to slow flowing hydrosystems, it is often epiphytic to algae and macrophytes, and has the capability of colonising artificial drainages and canals, slow streams, irrigation and drainage ditches and polluted open sewers (Lai *et al.* 2009; Málnás *et al.* 2016; Morhun *et al.* 2021).

H. europaea was first described as *Helobdella striata* from Germany (Kutschera 1985), but the latter name was preoccupied and this species was renamed (Kutschera 1987). Since then it has been reported in other European countries including the Netherlands (van Haaren *et al.* 2004), Spain (Jueg 2008; Reyes-Prieto *et al.* 2014), Hungary (Málnás *et al.* 2016) and more recently Ukraine (Morhun *et al.* 2021). Besides Europe, it is also present in California (Kutschera *et al.* 2013), Taiwan (Lai *et al.* 2009), North Africa (Mabrouki *et al.* 2019), South Africa, Hawai'i, New Zealand (Siddall and Budinoff 2005), and Australia (Govedich and Davies 1998 – referred to as *H. papillornata*; Pfeiffer *et al.* 2004; Siddall and Budinoff 2005).

Freshwater leech studies in Fiji are still in their infancy, with previous work being limited to the identification of native specimens in the family Salifidae (Rashni 2013; Rashni 2014*a*, 2014*b*; Rashni 2015). The presence of *Helobdella*. cf. *europaea* in Fijian river systems on the island of Viti Levu is the first record of this species in the country. Individuals have been observed on three occasions in Fijian waterways: in 2015 during an environmental impact assessment study at Qalinabulu Creek, Bavu village, Nadroga Province; in 2015 and 2019 at Balevuto village, Ba Province; and in 2019 in the upper- and mid-Wainamau sub-catchment waterways (Rashni 2020). This last field visit in 2019 by authors to Koroboya village, Ba Province led to *in situ* population observations and preliminary live specimen morphological verification using established descriptions (Kutschera 2004; Lai *et al.* 2009; Kutschera *et al.* 2013). Anecdotal reports suggest the presence of *H.* cf. *europaea* has been known to local communities for at least a decade (Semisi Qamese, pers. comm., 2015).

Considering that (1) *H. europaea* is a recognised invasive species also recorded from neighbouring Australia and New Zealand (GBIF 2020), and (2) it parasitises aquatic invertebrates (Kutschera 2004; Málnás *et al.* 2016); it is important to confirm the taxonomic identity of Fijian *Helobdella* leech specimens. Therefore, the current research reports on the taxonomic verification of Fijian *H.* cf. *europaea*, and presents the complete mitochondrial genome of this species, which can be used as a source tracking tool should it spread to new localities. These data will inform interventions relating to possible ecosystem health impacts associated with this leech, both in Fiji and where occurrences are reported elsewhere.

Methodology

Study area and sample collection

A kick-netting technique (Stark *et al.* 2001) was used to collect leech specimens (n = 50) for genetic analyses from the Koroboya village stream system, Ba Province, Viti Levu, Fiji in 2020 (Fig. 1). The specimens were preserved whole in 80% ethanol.

Morphological analyses

Additional specimens obtained from Bavu village in the Nadroga-Navosa province (year 2015) and Ba province (years 2015, 2019, and 2020, n = 52) were morphologically identified as *H. europaea* following Kutschera (2004), Lai *et al.* (2009) and Kutschera *et al.* (2013). Examinations involved measuring specimen sizes (body length at rest), observing form (body shape, presence of a convex dorsum, anterior sucker, posterior (caudal) sucker and pair of eyes with annulation along the axis of the body), colour and pattern.

Phylogenetic analyses

Total DNA was extracted from individual whole leech specimens (n = 32/50 specimens collected) using a CTAB (cetyltrimethylammonium bromide) chloroform/isoamyl alcohol protocol (Adamkewicz and Harasewych 1996), modified as per Lal *et al.* (2016) at the University of the South Pacific, Suva, Fiji. Purified DNA was quantified with a QUBIT flourometer (Life Technologies, MA, USA) using the Broad Range DNA assay. Subsequently, 16 samples were further processed for Polymerase Chain Reaction (PCR) and Sanger sequencing at the School of Natural Sciences at Massey University in New Zealand.

A 658 bp mitochondrial cytochrome *c* oxidase subunit I (*cox1*) DNA fragment was targeted for amplification using the universal primers (Folmer *et al.* 1994): LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). Amplification was carried out on a Whatman T1 (Whatman Biometra, Göttingen, Germany) thermal cycler, with each reaction using EmeraldAmp GT Master Mix (Takara, Kusatsu, Shiga, Japan), 10 pmol of each primer and 2 ng template DNA. Cycling conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 40 s, and a final extension step at 72°C for 5 min with a hold at 10°C. PCR products were assayed using 1.5% agarose gel electrophoresis before clean-up with a SAP-Exo kit (Jena Bioscience, Jena, Germany). Bi-directional sequencing was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Waltham, MA, USA) following standard instructions.

Chromatograms were edited using CodonCode Aligner ver. 3.7.1–6.0.2 (CodonCode Co., Centerville, MA, USA) in

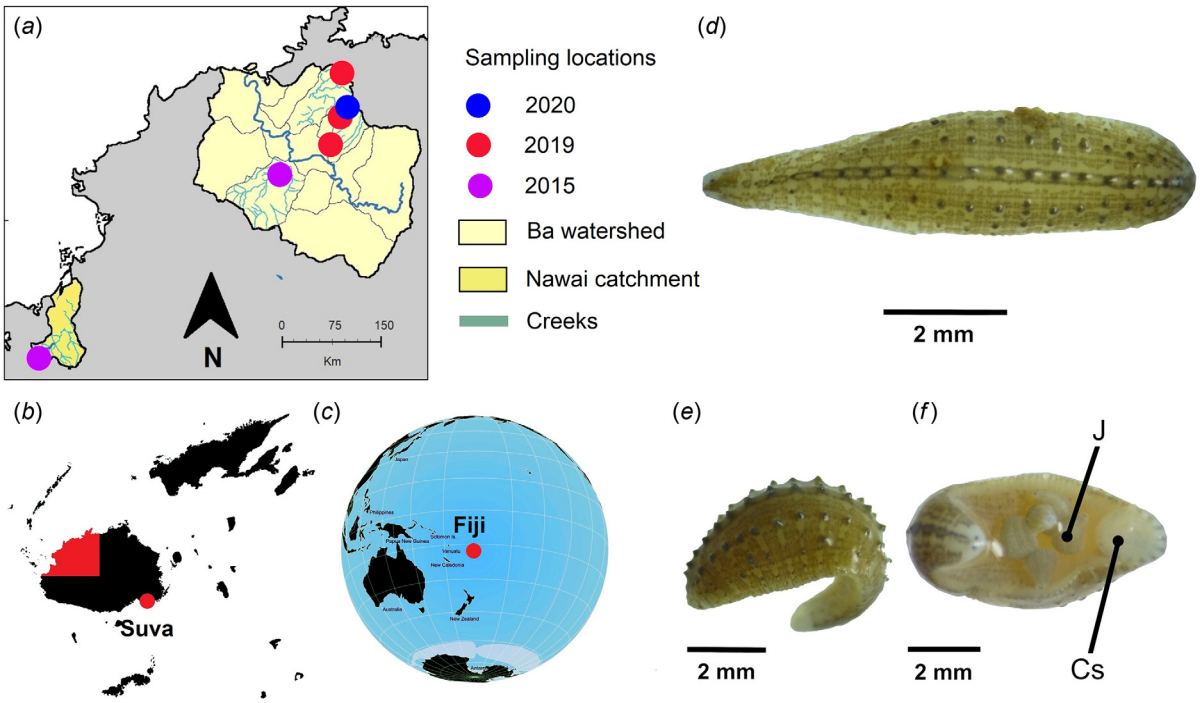

Fig. 1. Collection locations and representative specimens of *H.* cf. *europaea* from Fiji. (*a*) Koroboya village stream system and wider Ba Province watershed annotated with coloured sampling locations and dates, (*b*) the location of Ba Province (red) in Fiji and (*c*) Fiji relative to the South Pacific region. Fijian specimen features shown include a (*d*) dorsal view with five rows of distinct black-tipped papillae; (e) cone-shaped dorsal papillae, dark longitudinal stripes and white chromatophores; and (*f*) ventral view with fully developed young attached – (J), a pair of eyes with (Cs) caudal sucker. Insets (*a*–*c*) were generated using QGIS v 3.18.3-Zürich and open source data obtained from the Humanitarian Data Exchange (https://data.humdata.org/dataset/cod-ab-fji) and Natural Earth (https://www.naturalearthdata.com/).

the Geneious v.9.0.4 package (Kearse *et al.* 2012). Gaps in the sequences were treated as missing data, and final alignment for sequences was by MUltiple Sequence Comparison by Log-Expectation (MUSCLE, (Edgar 2004). The final alignment was subjected to substitution model testing using jModelTest (Posada 2008), with Bayesian Information Criterion (BIC) scores including the negative log likelihood (−lnL) and BIC difference (delta parameter) used to select the optimal model. Edited and aligned forward and reverse sequences were then subjected to a megaBLAST search to retrieve matching *cox*1 *Helobdella* sequences from the NCBI GenBank (Table 1). Sequences belonging to *Placobdella phalera* and *Glossiphonia elegans* were used as outgroups as per Lai *et al.* (2009).

Bayesian inference was used for phylogenetic reconstruction in the MrBayes v3.2 package (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist *et al.* 2012). Parameter settings for the data block were set as follows: lset applyto = (all), nst = 2, rates = gamma; prset applyto = (all), lset applyto = (all). Each analysis incorporated two runs of 100 000 000 generations, with four independent chains for each run, a temperature of 0.10 for the heated chains, sampling frequency of 1000 and burn-in fraction of 20%. The burn-in threshold was selected on the basis that both independent runs had achieved stationarity (i.e. stable log likelihood values reached for all sampled trees, indicated by the average standard deviation of split frequencies <0.01). Convergence was also assessed using Tracer v.1.6 independently (Rambaut *et al.* 2003). Final trees were retained by selecting only those post-burn in trees producing the highest individual (p) and cumulative posterior (P) probabilities during Markov Chain Monte Carlo (MCMC) computations, of $p = 0.000$ and $P \geq 0.995$ respectively. A consensus tree was then constructed from the final credible sets of trees using the strict consensus method in Dendroscope 3.5.7 (Huson *et al.* 2007). All phylograms were visualised, inspected and edited in FigTree (https://tree.bio.ed.ac.uk/) v.1.4.2 (Rambaut 2014). Net evolutionary divergence estimates between the different *Helobdella* sp. taxa, as well as among *H. europaea* sampled from different collection sites were computed in MEGA6 (Tamura *et al.* 2013). Standard error estimates were obtained following a bootstrap procedure with replication.

The mitochondrial genome of *H. europaea*

Ten nanograms of *H. europaea* total genomic DNA from a Fijian specimen and 62.5 μM of the selective multiple

Table 1. Specimen details for 80 leech sequences analysed.

Species	GenBank accession number	Collection location	Reference
H. europaea	AF329052	Magill Creek, Brisbane, Australia	Reyes-Prieto *et al.* (2014)
H. europaea	AY856047	Aura Vale Lake, Australia	Reyes-Prieto *et al.* (2014)
H. europaea	AY856048	South Africa	Reyes-Prieto *et al.* (2014)
H. europaea	AY856049	New Zealand	Reyes-Prieto *et al.* (2014)
H. europaea	DQ995297	Galt, California, USA	Reyes-Prieto *et al.* (2014)
H. europaea	DQ995298	Galt, California, USA	Reyes-Prieto *et al.* (2014)
H. europaea	DQ995304	Berkeley, California, USA	Bely and Weisblat (2006)
H. europaea	FJ000349	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. europaea	FJ000350	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. europaea	FJ000351	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. europaea	FJ000352	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. europaea	KC904241	Castellon, Valencia, Spain	Reyes-Prieto *et al.* (2014)
H. europaea	KC904242	Castellon, Valencia, Spain	Reyes-Prieto *et al.* (2014)
H. europaea	KC904243	Castellon, Valencia, Spain	Reyes-Prieto *et al.* (2014)
H. europaea	KU738724	Debrecen, Hungary	Langguth and Kutschera (2016) in Morhun *et al.* (2021)
H. europaea	MF804537	Mississippi, USA	Richardson *et al.* (2017)
H. europaea	MG976140	Victoria, Australia	Carew *et al.* (2018)
H. europaea	MN335875	Spain	Perera *et al.* (2019)
H. europaea	MT258557	Kharkiv, Ukraine	Morhun *et al.* (2021)
H. modesta	AF329040	Ohio, USA	Reyes-Prieto *et al.* (2014)
H. modesta	HQ179853	Washington, USA	Reyes-Prieto *et al.* (2014)
H. modesta	HQ179854	Washington, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319988	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319989	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319990	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319991	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319992	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319993	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319994	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319995	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. modesta	JF319996	Connecticut, USA	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000342	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000343	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000344	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000345	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000346	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000347	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	FJ000348	Taipei, Taiwan	Reyes-Prieto *et al.* (2014)
H. octatestisaca	HQ179855	Querétaro, Mexico	Reyes-Prieto *et al.* (2014)
H. octatestisaca	HQ179856	Ameca, Jalisco, Mexico	Reyes-Prieto *et al.* (2014)
H. octatestisaca	HQ179857	Hidalgo, Mexico	Reyes-Prieto *et al.* (2014)
H. octatestisaca	HQ179858	Guanajuato, Mexico	Reyes-Prieto *et al.* (2014)
H. octatestisaca	HQ179859	Tabasco, Mexico	Reyes-Prieto *et al.* (2014)

(*Continued on next page*)

Table 1. (*Continued*).

Species	GenBank accession number	Collection location	Reference
H. octatestisaca	HQ179860	South Africa	Reyes-Prieto *et al.* (2014)
H. papillata	AF329042	Michigan, USA	Reyes-Prieto *et al.* (2014)
H. papillata	AF329043	Michigan, USA	Reyes-Prieto *et al.* (2014)
H. papillata	AF329046	Virginia, USA	Reyes-Prieto *et al.* (2014)
H. papillata	KP176607	USA	Richardson *et al.* (2015)
H. papillata	MK416023	USA	Beresic-Perrins *et al.* (2019) in Morhun *et al.* (2021)
H. papillata	MK416024	USA	Beresic-Perrins *et al.* (2019) in Morhun *et al.* (2021)
H. papillata	MK416025	USA	Beresic-Perrins *et al.* (2019) in Morhun *et al.* (2021)
H. robusta	DQ995299	California, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995300	California, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995301	California, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995302	California, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995306	Austin, Texas, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995307	Austin, Texas, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995308	Austin, Texas, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995309	Austin, Texas, USA	Reyes-Prieto *et al.* (2014)
H. robusta	DQ995310	Austin, Texas, USA	Reyes-Prieto *et al.* (2014)
H. socimulcensis	DQ995311	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179866	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179867	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179868	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179869	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179870	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179871	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	HQ179872	Mexico	Reyes-Prieto *et al.* (2014)
H. socimulcensis	MG821615	Mexico	Bely and Weisblat (2006)
H. socimulcensis	MG821616	Mexico	Tessler *et al.* (2018)
H. socimulcensis	MK208676	Mexico	Jiménez-Armenta and Oceguera-Figueroa (2019)
H. socimulcensis	MK208677	Mexico	Jiménez-Armenta and Oceguera-Figueroa (2019)
H. socimulcensis	MK208678	Mexico	Jiménez-Armenta and Oceguera-Figueroa (2019)
H. socimulcensis	MK208679	Mexico	Jiménez-Armenta and Oceguera-Figueroa (2019)
H. socimulcensis	MK208680	Mexico	Jiménez-Armenta and Oceguera-Figueroa (2019)
H. triserialis	AF329054	Bolivia	Siddall and Borda (2003)
H. triserialis	DQ995303	USA	Bely and Weisblat (2006)
H. triserialis	KC771417	USA	Schmerer *et al.* (2013)
Outgroups			
Placobdella phalera	AF003278		Siddall and Burreson (1998)
Glossiphonia elegans	AF003258		Siddall and Burreson (1998)

Species, collection location, sequence accession data and citing references are reported.

displacement amplification (MDA) primers TATT*A*A and TAAT*T*A (* indicates exonuclease resistant phosphorothioate bonds) were denatured in 5 μL of 1× EquiPhi Reaction Buffer at 95°C for 3 min. The mix was cooled on ice for 3–5 min then added to 20 μL of 1× reaction mix containing EquiPhi29 DNA polymerase Reaction Buffer, DTT, dNTPs and EquiPhi29 DNA Polymerase as per the protocol: https://www.thermofisher.com/order/catalog/product/A39390. The reaction was incubated at 37°C for 3 h followed by an inactivation step at 65°C for 10 min.

After whole genome amplification, impurities were removed from the amplified DNA using a DNA Genomic Clean and Concentrator Column (Zymo Research). The amplified DNA was eluted from the column in 15 μL of water and the concentration of the eluate (290 ng/μL) determined by QUBIT (ThermoFisher). A MinION PCR-free library was then prepared using the Oxford Nanopore Technologies (ONT) rapid barcoding kit (SQK-RBK004) and sequenced on a MinION flongle (R9.4.1) using an ONT Mk1C MinION device running MinKNOW v21.10.8. *H. europaea* total genomic DNA was also sequenced without MDA on a flongle on the ONT Mk1C MinION device.

Bases were called using Guppy v5.0.17. Adaptors were trimmed using Porechop v0.2.4 (Wick 2018). Assembly of the mitochondrial genome was made using Trycycler (Wick *et al.* 2021). Short and low-quality reads were removed with NanoFilt https://github.com/wdecoster/nanofilt to produce two filtered read sets: (1) reads of length ≥1000 bases and (2) reads of length ≥5000 bases. BLAST*n* searches https://anaconda.org/bioconda/blast of read sets against the mitochondrial genome *of Placobdella lamothei* NC_030269.1 and against the assembled *H. europaea* mitochondrial genome were used to identify and count mitochondrial reads in MDA enriched and non-enriched DNA. Flye v2.9 was used to assemble contigs from read sets https://anaconda.org/bioconda/flye, Minimap2 v2.24 was used to map reads to linear contigs https://anaconda.org/bioconda/minimap2 and Geneious Prime 2022.2.1 www.geneious.com to map reads to a circular reference. Mapping to a circular reference was necessary as reads that spanned the 5′ and 3′ ends of the assembled mitochondrial genome were not well mapped using a linear mapper.

Comparing the counts of mitochondrial DNA (mtDNA) reads in amplified and unamplified DNA allowed us to calculate the amount of enrichment of mtDNA produced by selective MDA. Annotations to the mitochondrial genome were made using Geneious 9.1.8 https://www.geneious.com and these were compared to mitochondrial genome sequences for *P. lamothei* NC_030269.1 and *Helobdella robusta* AF178680.1. The mitochondrial genome sequence of H. europaea is available from the NCBI GenBank under accession number: NC_072606.1.

Ethical standards

Responsibility for the ideas expressed in this paper rest with the authors alone. The authors assert that all procedures contributing to this work comply with applicable national and institutional ethical guidelines on the care and use of laboratory or otherwise regulated animals.

Results

Morphological analyses

Preserved adult specimens are depicted in Fig. 1*d–f*. Microscopic observations of morphological features revealed visible segments (Fig. 1*d*), a prominent 'bumpy' dorsal surface with distinguishing features (Fig. 1*e*) and a suction disc at the tail end (Fig. 1*f*). Other features observed included a moderately flattened ovate-lanceolate body, one pair of punctiform to triangular eyes in segment III (3rd annulus), five rows of distinct, black-tipped papillae of the dorsum, dorsal pigmentation arranged in numerous longitudinal dark grey stripes and the absence of a nuchal scute. All specimens collected from Koroboya village stream had a size range of 3–8 mm (Rashni and Brown 2021) and features were concordant with *H. europaea*.

Ecological characteristics

The Fijian specimens collected from Ba and Nadroga provinces inhabit slow flowing freshwater streams with heavily modified riparian vegetation. The Koroboya stream is 3–5 m wide and the channel depth range is 0.10–1.0 m with a silted streambed. The streambed was mostly bare during sampling and some areas were covered with *Chara* sp. (a green macroalga) and leaf litter. Large numbers of leeches were found on silt covered *Chara* sp. leaves and instream leaf litter dominated by *Mangifera indica* (mango) leaves. Previously, *Helobdella* leeches were found on silted *Potamogeton crispus* (a freshwater aquatic plant) beds in the Qalinabulu creek, Balevuto village, Nadroga.

Phylogenetic analyses

Sixteen samples of leech total DNA from Fiji amplified and were sequenced successfully, returning identical sequences. A megaBLAST search produced 100% identity matches to 12 catalogued *H. europaea* sequences: *AY856049.1*, *DQ995298.1*, *AF329052.1*, *DQ995297.1*, *MF804537.1*, *AY856048.1*, *MG976140.1*, *FJ000349.1*, *KC904241.1*, *KC904242.1*, *MN335875.1* and *DQ995304.1*. Given that the 16 Fijian samples contained identical sequences, a consensus sequence (Supplementary Material 3) was generated for further analyses.

The consensus Fiji sequence was pooled with 80 *Helobdella* and outgroup sequences (Table 1) for alignment and substitution model testing. The Hasegawa–Kishino–Yano model with gamma-shaped rate variation and invariable sites (HKY + G + I) was determined to have the best fit from jModelTest results (−ln L = 2076.5, BIC = 5835.7 and AICc = 4491.6 [corrected Akaike information criterion]), and was subsequently used for Bayesian reconstruction of phylogenetic relationships. Two parallel runs of MrBayes were used to generate the final tree, where the final average standard deviation of split frequencies achieved was 0.0028 with average potential scale reduction factor (PSRF) for parameter values remaining at 1.000. In total, 16 002/20 002 trees were sampled from the parallel MrBayes runs and a burn-in set (20%) discarded to generate an initial subset of 12 802 trees. Subsequently, 89 highly credible trees were retained

(posterior probability $p \leq 0.000$ and cumulative posterior probability $P \leq 0.995$) and a strict consensus final tree generated (Fig. 2).

Our reconstruction confirms the assignment of the Fijian leech samples to *Helobdella europaea*, as the consensus sequence resolved a strong monophyletic clade with *H. europaea* specimens from Australia, New Zealand, Spain, Taiwan, South Africa, Hungary, Ukraine and the USA. This clade has short internal branch lengths and high posterior probability node support (100). The *H. europaea* clade is paraphyletic within a larger grouping comprising the sister taxa *H. triserialis* and *H. socimulcensis*. Two other large paraphyletic groups were identified, highlighting relationships between *H. robusta* and *H. papillata*; and *H. modesta* with *H. stagnalis* and *H. octatestisaca*, respectively. All clades were resolved with high posterior probabilities.

Evolutionary divergence estimates

Relationships identified in the Bayesian tree topology were supported in the pairwise net evolutionary divergence estimates computed between taxa and collection locations (Tables 2 and 3). Pairwise divergence estimates between the Fijian *H. europaea* sample sequence and all other *H. europaea* sequences were effectively zero (Table 3), supporting conspecificity of these taxa. A minor degree of divergence was detected between the Fijian and Australian sequences (0.0015 ± 0.0011), however the Australian samples also reflected an identical degree of separation from other conspecific sequences from Spain, Taiwan, the USA, Hungary and South Africa. The largest but still small degree of separation was evident between the Ukrainian sequence (MT258557) and all others (0.0022–0.0037).

The close affinity of *H. europaea* with *H. socimulcensis* (0.0297 ± 0.0079) and *H. triserialis* (0.0304 ± 0.0086) provides support that these three taxa may comprise a species complex (Table 2, values in bold), which is further discussed by Morhun *et al.* (2021). Similarly, pairwise divergence estimates were low between *H. robusta* with *H. papillata* (0.0277 ± 0.0054), and *H. stagnalis* and *H. octatestisaca* (0.0084 ± 0.0035), respectively. Estimates were highest between *H. octatestisaca* and all other taxa with the exception of *H. stagnalis*, ranging from 0.2027 ± 0.0298 (*H. papillata*) to 0.2382 ± 0.0349 (*H. europaea*).

Mitochondrial genome of *H. europaea*

The genome sequence determined for *H. europaea* was assembled from high coverage of ONT MinION sequenced MDA mitochondrial DNA reads using Flye v. 2.9 based on the tutorial developed for Trycyler (Wick *et al.* 2021). Selective MDA using the protocol and primers described enriched mtDNA from 1% to more than 28% reads of the reads ≥1 kb in length. Details on enrichment and genome coverage have been provided in Supplementary Material 1 and 2.

The assembled annotated genome shown in Fig. 3 was found to be very similar in organisation to that of *P. lamothei* NC_030269.1 and *H. robusta* AF178680.1. The complete mitochondrial genome sequence is available online under accession NC_072606.1 (https://www.ncbi.nlm.nih.gov/nuccore/NC_072606.1) and as Supplementary Material 4. Unique to *H. europaea* was the presence of repeats in the control region. These repeats were identified in both amplified and unamplified *H. europaea* DNA that were sequenced on the MK1C minion device. It was not determined whether the number of repeats were variable within and between individuals of *H. europaea*.

Discussion

The independent morphological and molecular approaches employed confirm assignment of the Fijian leech samples to *H. europaea*, establishing the first record of this invasive annelid in the Pacific Islands region. The complete mitochondrial genome sequence reported here will also be a valuable resource for traceability studies involving spread of this species, and for future comparative analyses.

Morphological and ecological insights

The external morphology of samples collected in Fiji is consistent with descriptors for *H. europaea* reported in other studies (Kutschera 2004; Kutschera *et al.* 2013) and further studies examining specimen gut contents may reveal potential impacts on native aquatic invertebrate fauna. Our observations suggest that *H. europaea* in Fiji prefers soft-bottomed sections of streams inhabited by macrophytes (*P. crispus* and *Chara* sp.) and leaf litter.

Molecular barcoding

Resolution of the Fijian leech samples in a monophyletic clade, strong sequence identity matches and evolutionary divergence computations with catalogued *H. europaea* specimens confirm this species is now present in Fiji. Results reported here underscore the utility of molecular barcoding tools for identifying invasive or potentially invasive taxa. The *cox1* locus has successfully been used to identify *H. europaea* in various other parts of the world where concerns have been raised on its presence, including Ukraine (Morhun *et al.* 2021), Spain (Reyes-Prieto *et al.* 2014) and New Zealand (Siddall and Budinoff 2005).

Molecular barcoding approaches have further characterised evolutionary relationships in the genus *Helobdella*, which has provided much needed taxonomic clarity. Siddall and Budinoff (2005) validated the current species name of *H. europaea*, after reporting this leech was originally named *H. striata* based on specimens collected in Germany and subsequently independently described as *Helobdella papillornata* from Australia – see Govedich and Davies (1998). Given that *H. europaea* is endemic to South America and affinities

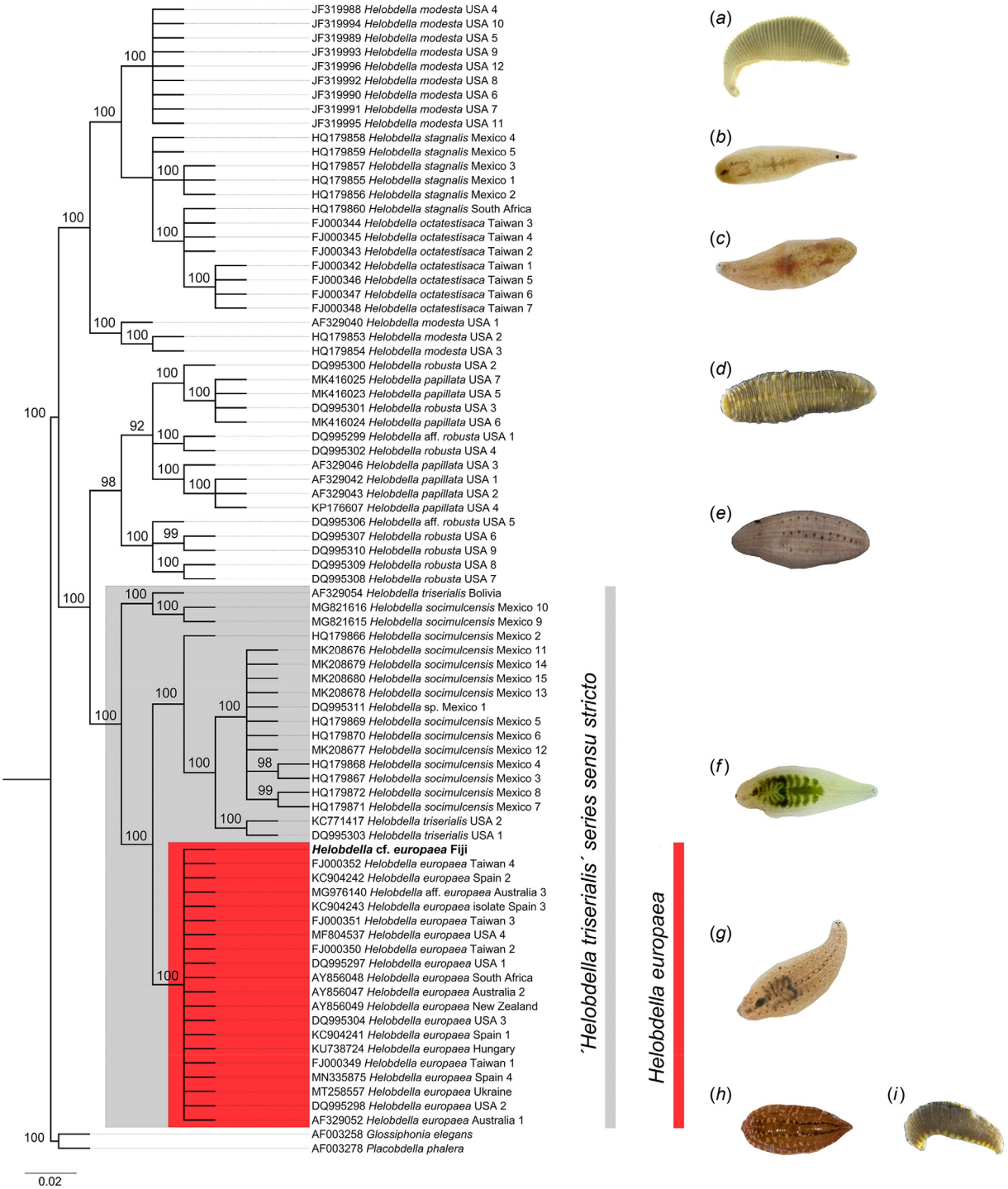

Fig. 2. Bayesian phylogenetic reconstruction based on 81 *cytochrome c oxidase* (*cox1*) nucleotide sequences. A consensus sequence was generated for the Fijian samples from 16 individual sequences. The tree presented is a strict consensus of 89 highly credible trees (cumulative posterior $P \leq 0.995$). Node support values for major clades indicate posterior probabilities while the scale bar represents the number of substitutions per site. The major clade resolved for *Helobdella europaea* is highlighted in red, while the larger clade for the *H. triserialis* series *sensu stricto* as per Morhun *et al.* (2021) is presented in grey. Taxon images depict *H. modesta* (*a*), *H. stagnalis* (*b*), *H. octatestisaca* (*c*), *H. papillata* (*d*), *H. robusta* (e), *H. triserialis* (*f*) and *H. europaea* (*g*). Outgroup taxa used were *Glossiphonia elegans* (*h*) and *Desserobdella phalera* syn. *Placobdella phalera* (*i*). Images (*b*, *c*, *f–h*) are used with permissions from iNaturalist.org users mentioned in the acknowledgements section. The remaining images are adapted from www.boldsystems.org, with the following attributions: (*d*) and (*i*), no rights reserved/public domain; (*a*) and (e) CC BY CBG Photography Group.

Table 2. Estimates of net evolutionary divergence between groups of sequences for all taxa.

	H. robusta	*H. papillata*	*H. stagnalis*	*G. elegans*	*H. socimulcensis*	*P. phalera*	*H. triserialis*	*H. europaea*	*H. octatestisaca*	*H. modesta*
H. robusta	–	0.0054	0.0310	0.0366	0.0240	0.0353	0.0232	0.0280	0.0317	0.0274
H. papillata	**0.0277**	–	0.0293	0.0391	0.0247	0.0353	0.0237	0.0281	0.0298	0.0272
H. stagnalis	0.2039	0.1967	–	0.0408	0.0340	0.0372	0.0315	0.0329	0.0035	0.0318
G. elegans[A]	0.2579	0.2693	0.2757	–	0.0319	0.0294	0.0334	0.0354	0.0424	0.0339
H. socimulcensis	0.1480	0.1501	0.2230	0.2238	–	0.0303	0.0046	0.0079	0.0338	0.0232
P. phalera[A]	0.2443	0.2453	0.2566	0.2090	0.2061	–	0.0277	0.0322	0.0364	0.0316
H. triserialis	0.1316	0.1323	0.2049	0.2234	**0.0041**	0.1818	–	0.0087	0.0323	0.0221
H. europaea	0.1733	0.1698	0.2198	0.2391	**0.0297**	0.2174	**0.0304**	–	0.0349	0.0252
H. octatestisaca	0.2150	0.2027	**0.0084**	0.2901	0.2284	0.2584	0.2159	0.2382	–	0.0323
H. modesta	0.1816	0.1765	0.2062	0.2244	0.1485	0.2210	0.1381	0.1667	0.2094	–

The number of base substitutions per site from estimation of net average between groups of sequences are shown. Standard error estimates are shown above the diagonal in blue and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tamura–Nei model (Tamura and Nei 1993) with the rate variation among sites modelled with a gamma distribution (shape parameter = 1). The analysis involved 81 nucleotide sequences, all positions containing gaps and missing data were eliminated with a total of 414 positions in the final dataset. Analyses were conducted in MEGA6 (Tamura *et al.* 2013).
[A]Outgroup taxa.

Table 3. Estimates of net evolutionary divergence between groups of sequences for *H. europaea* segregated by sampling location.

	Spain	Taiwan	Australia	USA	Hungary	South Africa	Fiji	New Zealand	Ukraine
Spain	–	0	0.0011	0	0	0	0	0	0.0022
Taiwan	0	–	0.0011	0	0	0	0	0	0.0022
Australia	0.0015	0.0015	–	0.0011	0.0011	0.0011	0.0011	0.0011	0.0024
USA	0	0	0.0015	–	0	0	0	0	0.0022
Hungary	0	0	0.0015	0	–	0	0	0	0.0022
South Africa	0	0	0.0015	0	0	–	0	0	0.0022
Fiji	0	0	0.0015	0	0	0	–	0	0.0022
New Zealand	0	0	0.0015	0	0	0	0	–	0.0022
Ukraine	0.0022	0.0022	0.0037	0.0022	0.0022	0.0022	0.0022	0.0022	–

The number of base substitutions per site from estimation of net average between groups of sequences are shown. Standard error estimates are shown above the diagonal in blue and were obtained by a bootstrap procedure (5000 replicates). Analyses were conducted using the Tamura–Nei model (Tamura and Nei 1993) with the rate variation among sites modelled using a gamma distribution (shape parameter = 1). The analysis involved 20 nucleotide sequences, all positions containing gaps and missing data were eliminated with a total of 414 positions in the final dataset. Analyses were conducted in MEGA6 (Tamura *et al.* 2013).

are apparent with other South American *Helobdella* spp., and also that unfortunately the appropriate name of *Helobdella* (*triserialis*) *lineata* is already assigned to a separate North American species, this name is valid (Siddall and Budinoff 2005).

Several studies have suggested that *H. europaea* is part of a species complex, which includes *H. triserialis* and *H. socimulcensis* (Siddall and Borda 2003; Siddall and Budinoff 2005; Morhun *et al.* 2021). Reconstructions generated during the current study show support for this *H. triserialis* complex, given the paraphyly of all *H. europaea* sequences included with *H. triserialis* and *H. socimulcensis* sequences for comparison.

The mitochondrial genome of *H. europaea*

The genome sequence determined for *H. europaea* is the first mitochondrial genome reported for the species and genus. Our MDA protocol and primers have also been used to achieve similar levels of mitochondrial (mt) genome enrichment with *Papilio* (butterfly) DNA, and we anticipate the protocol is applicable for mt genome enrichment in other invertebrates. Assembly and length determination of the control region in *H. europaea* was challenging, as this region comprises repeat sequences that were a feature of both MDA amplified and unamplified DNA. Dinucleotide repeats also occurred that are also a feature of the control region in *Placobdella lamothei* (NC_030269). Variation in the length of control region repeats has previously been observed in other animal species (Munwes *et al.* 2011), and it is possible that variation in the length of the control region also exists within individuals and populations of *H. europaea*.

The selective multiple displacement amplification and rapid barcoding Oxford Nanopore sequencing protocols

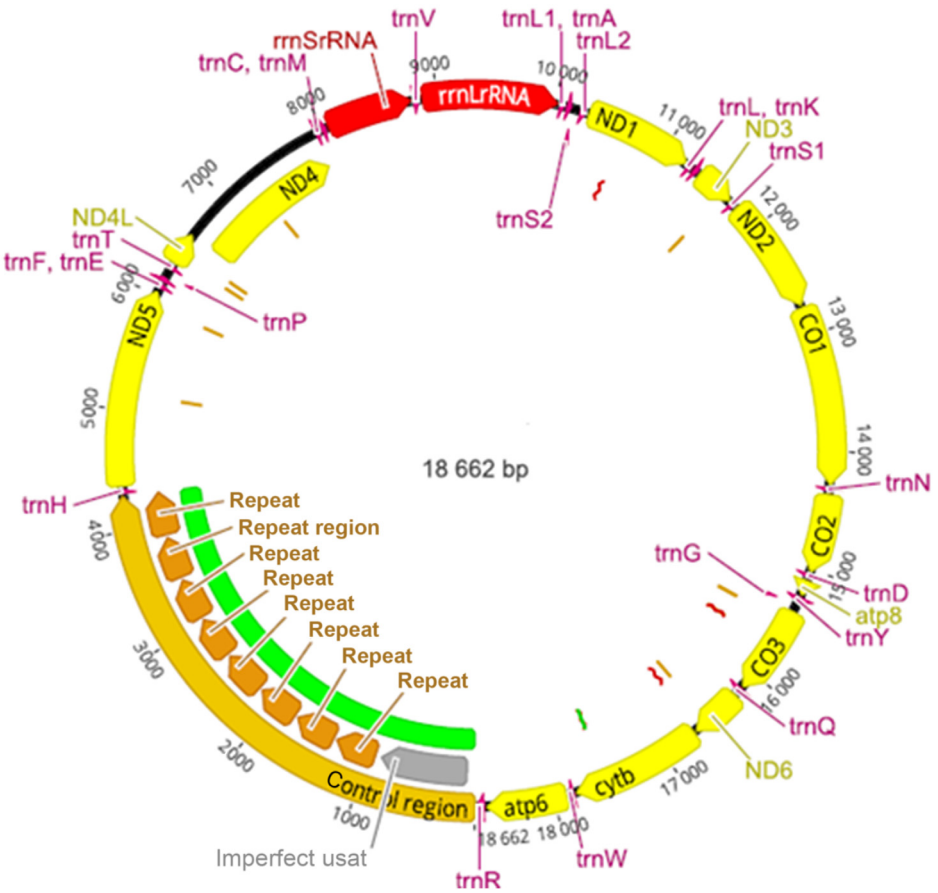

Fig. 3. Annotated mitochondrial genome of *Helobdella europaea*.

used to sequence the mitochondrial genome of *H. europaea* provide a cost-effective means for sequencing the mitochondrial genomes of invertebrates. These protocols require a heating block and a microcentrifuge, but are PCR-free (i.e. do not require a PCR machine). Thus the protocol has potential applications for biodiversity and biosecurity assessments in a low infrastructure setting.

Implications for watershed management

The presence of *H. europaea* in the Ba River catchment is concerning given the potential negative impacts (Kutschera 1989; Kutschera 2004; Pfeiffer *et al.* 2004) this organism might have on the native invertebrate fauna of Fiji. Several area-endemic taxa are present in the Ba River catchment, with aquatic insects, molluscs and worms (Kutschera 2004; Málnás *et al.* 2016) potentially being vulnerable. A total of 73 freshwater macroinvertebrate taxa have been recorded from this catchment area, of which 14% are confirmed as endemic to Fiji with a further 47% being possibly endemic pending confirmation (Rashni 2020). These endemic taxa include five caddisflies (*Abacaria fijiana*, *Abacaria ruficeps*, *Anisocentropus fijianus*, *Goera fijiana* and *Oxyethira fijiensis*), a damselfly, *Nesobasis* spp., a shrimp (*Caridina fijiana*), a micro-water strider *Fijivelia* sp., a water cricket (*Hydropedecticus vitiensis*) and spring snails *Fluviopupa* spp. The presence of *H. europaea* may pose the greatest potential risk to area-endemic spring snails belonging to the genus *Fluviopupa* (Gastropoda: Tateidae), which are of high conservation significance. Currently a total of 28 *Fluviopupa* species are recorded from Fiji, all of which are area- and national-endemics (Zielske and Haase 2014), and are included in the Fiji Endangered and Protected Species (EPS) Act 2017.

The likely mode of introduction of *H. europaea* to Fiji remains unknown at the present time, and a thorough bioassessment of this species across the connected riverine network of both the Ba and Nadroga provinces is strongly recommended to understand the extent of spread and potential impacts on resident aquatic biodiversity. In this regard for Fiji and elsewhere this species may occur, the newly determined mitochondrial genome sequence for *H. europaea* provides a genome reference for higher resolution comparative analyses and source tracking. Such data will further assist the Fiji Invasive Species Taskforce (FIST) in their decision making for evaluating the status of *H. europaea* in Fiji.

Although *H. europaea* has been recognised as an invasive species globally (Kutschera 2004; Málnás *et al.* 2016; Morhun *et al.* 2021) and is alien to South Pacific Island countries, at

this stage it can only be given the status of an introduced species in Fiji according to the corroborating definitions of Invasive Alien Species (IAS) via the Fiji National Biodiversity Strategies and Action Plans (NBSAPs), Convention on Biological Diversity (CBD) and the IUCN (International Union for Conservation of Nature) (Government of Fiji 2014; IUCN 2018; Department of Environment and Government of Fiji 2020). According to the Fiji NBSAP and CBD definitions, IAS are organisms found outside of their native geographical ranges that have spread and become invasive in their new habitats, and cause harm to biodiversity and other things that humans value (Government of Fiji 2014; Department of Environment and Government of Fiji 2020).

Results presented here offer a foundation on which further research assessing the extent of spread, ecology and ethology of *H. europaea* in Fiji and elsewhere may be based. In Fiji, outcomes of these future studies will further support the Biosecurity Authority of Fiji, the Ba and Nadroga-Navosa Provincial Administration and the Ba and Nadroga Yaubula Management Support Team to implement containment response protocols and formulate eradication strategies when/if required. Regionally in the Pacific and also globally, through the availability of the complete mitochondrial genome sequence for this species via this study, diagnosing its spread to other regions through traceability investigations is now possible.

Supplementary material

Supplementary material is available online.

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