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Research Article

Cryptic sympatric species across the Australian range of the global estuarine invader *Ficopomatus enigmaticus* (Fauvel, 1923) (Serpulidae, Annelida)

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Abstract

Ficopomatus enigmaticus (Fauvel, 1923) is a reef-building serpulid polychaete that has invaded estuaries worldwide, causing environmental and economic harm. Although Australia has long been suggested as a place of origin for the species, this remains unclear. We tested for genetic patterns across the range of *F. enigmaticus* in southern Australia, predicting that if the species is an Australian native, it would show evidence of (east-west) phylogeographic patterns often observed in native marine species in southern Australia. Unexpectedly, concordant patterns from mitochondrial (*Cyt B*) sequencing and nuclear marker (iSSR) profiles suggested the presence of at least three genetic groups (putative species), not distributed simply as "east" or "west". Two common (and closely related) groups were present across Australia and were often found together in the same aggregations. A third group was only found in southeast Australia and was morphologically similar to *F. uschakovi* (Pillai, 1960), a species previously reported from tropical areas. The discovery of multiple cryptic species with overlapping ranges means that more work is needed to resolve whether any of the *F. enigmaticus sensu lato* group has an Australian origin and to determine how they are related to invasive populations of *F. enigmaticus* elsewhere.

Key words: cryptic species, invasions, Serpulidae, Annelida, estuaries

Introduction

Bioinvasion can be the ultimate consequence of aquatic species being translocated around the world via hulls or the ballast water of ships (Carlton 1985, 1996a). While ever increasing frequencies and shorter transit times of oceanic vessels are accelerating the transport of species across the globe, human mediated establishment of non-native aquatic organisms has a long history, coincident with that of shipping (Reise et al. 1998; Hewitt et al. 2004). In some cases, species have spread so widely and/or been established for so long that it is now not clear where their original range was (Carlton 1996b). *Ficopomatus enigmaticus* (Fauvel, 1923), a serpulid polychaete, appears to be one such species that has invaded many of the world's warm temperate estuaries, likely over the last century. In many places, this worm forms reefs of calcareous tubes that have significant effects on estuarine ecosystems and it has become a nuisance biofouling organism (Davies et al. 1989). Although the species is now established around the world and unlikely to be eradicated (reviewed by Dittmann et al. 2009), there has been a long standing debate about where the species originates.

As its specific name suggests, the origin and invasion pathways of *F. enigmaticus* are unclear, but it is almost certainly not a native of France where it was originally described (Fauvel 1923). The author reasoned that the species was introduced to Europe because it forms conspicuous reefs up to several meters in diameter, which were unlikely to have

escaped attention from biologists working in Europe before 1900's. The original description in France coincided with the earliest observations of *F. enigmaticus* in Australia, which has led to long-standing speculation that the species might be an Australian native; Dew (1959) noted seeing *F. enigmaticus* in Cook's River in Sydney in 1910 and Allen (1953) mentioned that *F. enigmaticus* was widely spread around southern Australia in the early nineteen thirties. Both authors, along with others (e.g., Tebble 1953; Dixon 1981; Zibrowius and Thorp 1989) suggest that Australia was part of the native range of *F. enigmaticus*. Carlton (JT Carlton, Williams College, Williamstown, Massachusetts, USA, pers. comm.) argues that the only region of the world where the historical absence of *F. enigmaticus* cannot be excluded with confidence is Australia and points out that the center of *Ficopomatus* diversity lies in the Indo-West Pacific/Australasian region. In contrast, others (e.g., Pollard and Hutchings 1990; Hewitt 2002) have argued that *F. enigmaticus* is an introduced species in Australia. Unfortunately, both arguments are supportable. As the species was first noticed when it was apparently spreading in Europe, its appearance in Australian records might reflect the early stages of invasion into Australia as a result of increased shipping to both places from a non-Australian native range. Alternatively, the first records in Australia might have been delayed because no one had looked or known what to look for until that time. An absence of records before 1910 does not provide evidence that *F. enigmaticus* did not occur in Australia, as there are few historical records of any aquatic species in Australia (Ponder et al. 2002). Thus, determining whether southern Australia was part of the natural range of *F. enigmaticus* prior to European settlement requires other sources of information.

Paleontological data could provide insight into how long the species been present in Australia. For example, an absence of *Ficopomatus* tubes in Holocene sediments suggests *F. enigmaticus* as a recent addition to the estuarine fauna in Argentina (Schwindt and Iribarne 1998). While tubes of estuarine serpulids in southern Australia found in the Coorong estuary have been aged as at least 700 years old using ^{14}C techniques (Bone and Wass 1990), this does not necessarily mean those tubes were produced by *F. enigmaticus*. Certainly, extant populations of *F. enigmaticus* have been recorded in the same area by Geddes and Butler (1984) and substantial populations still exist in the Coorong now (Dittmann et al. 2009). Unfortunately, Bone and Wass (1990) neither provided any photographs, nor identified these fossil serpulids. So, while until now no other serpulids were recorded from southern Australian estuaries, an extremely slight possibility remains that these fossils are an extinct native species that has since been replaced by *F. enigmaticus*. Thus, although fossil evidence circumstantially supports a pre-European history in Australia, other lines of evidence are needed to assess whether *F. enigmaticus* is native.

Testing for geographic patterns of genetic diversity is another potential way to assess whether *F. enigmaticus* is an Australian native. Unfortunately, almost nothing is known about the genetic population structure of *F. enigmaticus* anywhere, except for a small allozyme-based study by Bertozzi et al. (2002), which found little genetic variation among three putatively invasive populations in Italy. In Australia, however, we would predict that if *F. enigmaticus* is native, then geographically structured patterns in genetic variation might have developed in south eastern Australia, similar to those found in other Australian marine species (e.g., Waters and Roy 2003; Waters et al. 2005, 2007; York et al. 2008; Ayre et al. 2009; Beck and Styan 2010; Waters et al. 2010). Although there are fewer examples of such structuring for estuarine species (e.g., Colgan and Schreiter 2011) and strong patterns of phylogeographic structuring may not necessarily occur for all native species in south eastern Australia (Colgan and da Costa 2009, 2013), such patterns are not known for invasive species in the same region (e.g., Andrew and Ward 1997; Mackie et al. 2006). In particular, we have previously detected strong patterns in genetic structuring for a closely related native intertidal serpulid across Bass Strait and between eastern and western Australian populations using mitochondrial Cytochrome B (*Cyt B*) sequence data (Styan et al. 2008). Separating either side of 80 Mile Beach in Bass Strait, Victoria (Styan, unpubl.), *Galeolaria caespitosa* Lamarck, 1818 and *G. gemineoa* Halt et al., 2009 were distinguished based on deep divergences on *Cyt B* sequences found in populations on the eastern *versus* southern coastlines of Australia (Halt et al. 2009). Given that both species of *Galeolaria* have near-continuous populations along the southern-Australian coastline and planktotrophic larvae capable of wide dispersal, we expected that genetic structuring among (native) populations of estuarine *F. enigmaticus* across the same coastline would show similar genetic structuring. Thus, we predicted that if *F. enigmaticus* was native to Australia, we would detect phylogeographic structuring within the species across its range in southern Australia.

We tested our hypothesis by looking at patterns of sequence variation in a mitochondrial marker (*Cyt B*) in southern Australia and among populations from the eastern and western parts of its range, which are

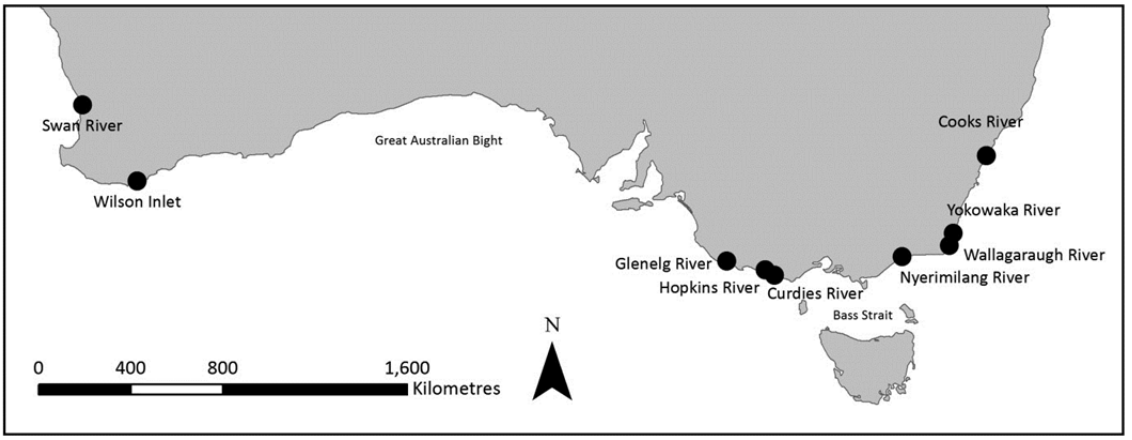

Figure 1. Distribution of estuaries sampled for *Ficopomatus enigmaticus sensu lato* along the southern-Australian coastline and other geographic sites mentioned in the text.

separated by large distances (~ 1000 km) along the southern coast of Australia (the Great Australian Bight) where there are no estuaries at all. Specifically, we predicted that haplotypes would be divergent between worms found in the western part of its range in Western Australia and the eastern part of its range in South Australia, Victoria, and New South Wales (Figure 1). In addition, we expected to find divergences in haplotypes and differences in their frequencies among populations within each of these regions, particularly across Bass Strait. Fundamentally, we expected to be assessing patterns of genetic divergence within/among populations of a single species (*F. enigmaticus*) or, possibly, two sister species each associated with western or eastern parts of the south eastern Australia (Halt et al. 2009). However, having unexpectedly found apparent structuring of haplotypes among and within some populations using *Cyt B* sequence data, we then tested whether these differences might reflect the presence of up to three sympatric (cryptic) species of *Ficopomatus* in southern Australia using an independent set of nuclear-based genetic markers (inter-sequence simple repeats; ISSRs).

Material and methods

Sites and sample collection

The sampling design, including sample sizes (n = 4–8 worms per site), was based on a previous study that detected strong genetic structuring in *Galeolaria* spp. using the same genetic marker (i.e., *Cyt B*; Styan et al. 2008). Populations of *F. enigmaticus* were collected from nine estuaries distributed across two well-known biogeographic boundaries in southern Australia: Bass Strait and Great Australian Bight (Figure 1; location details for collection sites are also given in the Supplementary material Table S1). As above, Bass Strait represents a biogeographic boundary for many marine invertebrates in south eastern Australia, potentially driven by contemporary coastal currents and/or historical glacial periods that created a land bridge between Tasmania and the Australian mainland that separated the southern and eastern temperate coastlines of Australia (Ayre et al. 2009). The Great Australian Bight is a region of ~ 1000 km of oceanic coastline along the central part of the southern coast of Australia that is devoid of permanent estuaries.

Worms were collected from both permanently open and seasonally-open closed estuaries and from sites likely to have experienced a range of shipping traffic, from none to very high. All samples used for genetic analysis were collected between November 2007 and May 2008. In southeast Australia (east of Bass Strait), the Cooks River (Co) runs through Sydney and connects to (the permanently open) Botany Bay near the largest international port in Australia. In contrast, the Yowaka River (Yo) and Wallagaraugh River (Wa) in southern New South Wales are small seasonally-open closed estuaries without ports or fishing harbours. The fourth site east of Bass Strait (Nyerimilang Creek; Ny) feeds into Lakes Entrance in Victoria, which is a moderately large estuary kept permanently open by dredging. All three estuaries in the area west of Bass Strait but east of the Great Australian Bight (Curdies River, Hopkins River, Glenelg River; Cu, Ho, Gl) are small seasonally-open closed estuaries and are not ports for shipping or fishing. In Western Australia (west

of the Great Australian Bight), Wilson Inlet (Wi) is a medium sized estuary and is seasonally-open closed. In contrast, the Swan River (Sw) is a large permanently open estuary connected to the Port of Fremantle, which is the major centre of shipping and fishing activity on the western side of the continent. Worms were collected from hard substrates such as rocks, logs, and jetty pylons. Where clumps/reefs occurred, a single small piece from a reef was collected. In the Wilson Inlet, Wallagaraugh River, Yowaka River and Nyerimilang Creek clumps/reefs were not found, but individual worms were collected from under rocks and logs in a relatively small area. Worms were either returned to the laboratory and stored at minus 20°C before sampling DNA onto Whatman FTA Elute Cards (Sigma Aldrich, USA) or processed directly onto FTA cards in the field.

DNA extraction, PCR amplification and sequencing

For outgroup comparison, we included two specimens representing congenerics *Ficopomatus miamiensis* (Treadwell, 1934) from Florida, USA (registered in South Australian Museum as SAM E3617) and *F. macrodon* Southern, 1921 from Samut Songkhram, Thailand (SAM E3618), see Kupriyanova et al. (2009). Genomic DNA was recovered from the FTA elute cards (and subsequently stored) following the manufacturer's instructions. The Polymerase Chain Reaction (PCR) was then used to amplify a fragment of the *Cytochrome Oxidase B* (*Cyt B*) gene using the primers Cytb 424F (5'-GGW TAY GTW YTW CCW TGR GGW CAR AT-3') (Boore and Brown 2000) and cobr825 (5'-AAR TAY CAY TCY GGY TTR ATR TG-3') (Burnette et al. 2005). Double-stranded PCR amplifications, using total genomic DNA as template, were performed in 25 µl reaction volumes containing 1 × PCR reaction buffer (Scientifix, Australia), 0.2 mM of each dNTP, 0.4 µM of each primer, 2 mM $MgCl_2$ and 0.5U of *Taq* DNA polymerase (Scientifix). PCR amplifications were carried out using the following PCR thermal-cycling profile: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 48 °C and 30 s at 72 °C, and an additional extension at 72 °C for 10 min. Amplified products were purified using PCRquick-spin purification kits (iNtRON Biotechnology, Inc., Korea).

Amplified PCR products were sequenced in both directions, using the same primers as in the PCR. DNA sequencing was carried out according to recommendations of Kieleczawa (2005): a mixture/aliquot containing 5–20 ng purified PCR product (final concentration) and 1 µM primer (final concentration) adjusted to a volume of 8 µl with 10mM Tris/0.01mM EDTA. A five minute heat-denaturation step at 98 °C, followed by placing the reaction mix on ice, was introduced prior to the addition of 2 µl of the ABI Big Dye Terminator v3.1 (Thermo Fisher Scientific Inc., USA) ready reaction mix. Consecutive runs of Ts (11 in a row) were used to overcome the problem of sequencing difficult templates (Kieleczawa 2005). The amplification reactions and purification of sequencing products then followed standard ABI Big Dye Terminator v3.1 cycle sequencing protocols. Sequencing products were analysed on a 3130 Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific Inc., USA).

Sequence chromatograms were viewed and edited in Sequence Scanner v1.0 (Applied Biosystems, Thermo Fisher Scientific Inc., USA) and the nucleotide sequence data was aligned in BioEdit v7.0.0 (Hall 1999). The alignment was pruned to remove primer sections of the fragment. Using the invertebrate mitochondrial code, the sequence data were translated to protein coding data to eliminate the possibility that the sequence data were nuclear paralogues.

Sequence alignment and phylogenetic analyses

All sequences were checked for contaminations with Blast Searches (Altschul et al. 1990). Sequences from both directions of target fragment were assembled using CodonCode Aligner ver. 3.6.1 (CodonCode Corporation, USA). The alignment was performed with ClustalW ver. 1.4 (Thompson et al. 1994) with default settings (10 gap opening penalty and 0.20 gap extension penalty). Sequences were subsequently edited by eyes and trimmed to equal length using BioEdit (Hall 1999). Approximately 266 bp were retained for phylogenetic and genetic structure analyses. Another serpulid, *Hydroides trivesiculosa* Straughan, 1967 (EU190476), was chosen as an outgroup to root the analyses.

Models for the sequence data partitions for Bayesian analysis were selected using MrModelTest ver. 3.7 (Posada and Crandall 1998) according to the hierarchical likelihood ratio tests (hLRTs). A different model was chosen for each of the three codons of the *Cyt B* sequence due to different evolution rates. The chosen models for codons one to three were K81uf+I+G, F81+G and TrN+G respectively.

Bayesian analyses were performed with MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) using selected models. Two simultaneous runs with four Markov chains each ran for 6000000 generations, sampling every 1000 trees. The first 1500000 generations (1500 trees) were discarded as burn-in. The majority rule consensus tree of the remaining 4500 trees for each analysis gave the posterior probabilities for each clade.

Both uncorrected pairwise distance and corrected pairwise distances among major clades using the Tamura-Nei model were calculated in MEGA 6 (Tamura et al. 2013).

ISSR PCR amplification

Given relatively large genetic distances among three broadly resolved groups from the mtDNA sequencing, we assessed whether these might represent species level differences by testing for (correlated) genetic differences using independent (nuclear-based) iSSR markers (Maltagliati et al. 2005; Cossu et al. 2012). Based on mtDNA sequences, we selected for ISSR screening a sub-set of 31 individuals, representing each of the three major groups identified by the phylogenetic analyses. Where possible, samples within each group were selected from across the three biogeographic regions (East of Bass Strait, West of Bass Strait, Western Australia) and replicate locations where both groups were present. Thus, the comparisons among clades included worms from different sequence groups that were found in both broad (i.e., within biogeographic region) and small scale (within population) sympatry. Individual worms re-sampled for iSSR banding patterns are indicated on the right side of the phylogram in Figure 2.

Following preliminary screening of a number of primers found on the web (http://www.biosci.ohio-state.edu/~awolfe/ISSR/protocols.ISSR.html), we selected two (UBC-818 $(CA)_8G$ and UBC-827 $(AC)_8G$) which could be consistently scored for this study. ISSR PCR amplifications, using total genomic DNA as template (as above), were performed in 12.5 µl reaction volumes containing 1 × PCR reaction buffer (Scientifix), 0.2 mM of each dNTP, 0.4 µM of primer, 2mM $MgCl_2$ and 0.25U of *Taq* DNA polymerase (Scientifix). ISSR PCR amplifications were carried out using the following PCR thermal-cycling profile: 3 min at 94 °C, 35 cycles of 30s at 94 °C, 30s at 50 °C and 120s at 72 °C, and an additional extension at 72 °C for 10 min. ISSR PCR products were analysed by electrophoresis using 1.5% agarose gel in 0.5X TBE Buffer. Gels were run for 1 hour at 100V, stained by soaking in a solution of ethidium bromide, and viewed under UV light. ISSR banding patterns were recorded by digital photography and later scored from image enhanced photographs. Loci were named for running lengths along the gel and where samples could not all be run on the one gel a random interspersion of samples/clades was done to prevent confounding. Re-runs/line-ups were also done to maintain scoring consistency within/among running gels.

Given that ISSRs are dominant diallelic markers, we constructed a matrix of similarity among individuals, based on a Jacquard index where loci were treated as a (presence/absence) variable for each individual. Although about half of the loci showed bands on only four individuals or fewer, all loci were included in the similarity matrix and subsequent analyses. All analyses were also repeated excluding uncommon loci (i.e., including only loci where bands were present in 15% or more of the samples) which did not change any of the outcomes (and so are not reported further).

Statistical analyses

Differences in the dissimilarities of iSSR banding patterns among the three sequenced based groups were compared using PRIMER v.6 (Clarke and Gorley 2006). The main comparison was a (one factor) test for differences in similarity among the three sequence based groups, using PERMANOVA (and PERMDISP) analyses, with similarities among individuals within groups as the error term; i.e., ignoring biogeographic regions or populations as factors. Having found a difference among groups, we used pairwise comparisons in PERMANOVA to identify which particular groups differed.

Results

Sequence alignment and phylogenetic analysis

A total of 48 individual specimens of *F. enigmaticus* from nine localities were sequenced for a fragment of the *Cyt B* gene. The sequences have been deposited in Genbank under accession numbers KP863736–KP863779. From these sequences we identified 11 haplotypes (Table 1). Within the complete alignment, including sequences representing *F. miamiensis*, *F. macrodon* and the outgroup *Hydroides trivesiculosa,* we identified variation in sequence length, which was always in the order of three base pairs (i.e., a single codon coding for an amino acid). We therefore translated the *Cyt B* sequences to amino acids and verified that variation in sequence length was due to insertion/deletions of entire codons (amino acids), enabling unambiguous alignment of all sequences. Translation of the *Cyt B* sequences to amino acids also suggested that the sequenced *Cyt B* mtDNA fragments were not nuclear paralogues, as there were no single base insertions or deletions and stop codons found (Zhang and Hewitt 1996). Consistent with other genetic studies on serpulids (Sun et al. 2012) sequence quality was variable and for the purposes of phylogeographic analyses the sequences were trimmed to equal length resulting in 266 bp length of the data matrix.

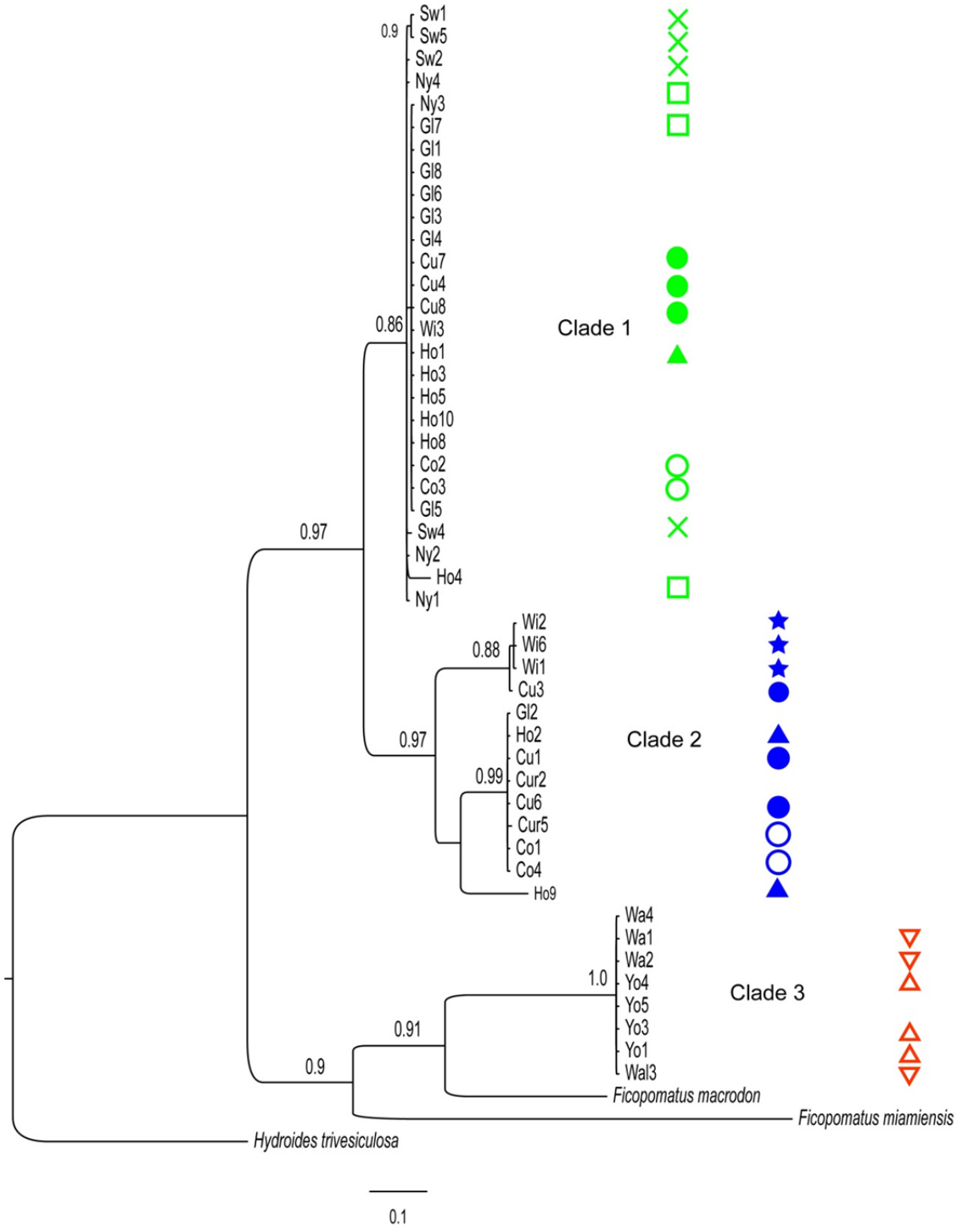

Figure 2. Bayesian 50% majority rule phylogram based on *Cyt B* sequences. Numbers above branches are posterior probabilities. Coloured symbols to the right of the diagram indicate worms that were subsequently resampled for iSSR banding patterns (and correspond to symbols in Figure 3). Collection location of samples is indicated by symbol type (Cooks River, hollow circles; Yowaka River, hollow upwards triangles; Wallagaraugh River, hollow downwards triangles; Nyerimilang River, hollow squares; Curdies River, solid circles; Hopkins River, solid upwards triangles; Wilson Inlet, star; Swan River, cross) while mtDNA group is indicated by symbol colour (1, green; 2, blue; 3, red).

Table 1. Number of occurrences of *Cyt B* haplotypes across biogeographic regions and locations.

	Group	1						2						3
	Haplotype	*A*	*B*	*C*	*D*	*E*		*F*	*G*	*H*	*I*	*J*		*K*
Biogeographic Region	**Sampling Location**													
East of Bass Strait	Cooks River			2						2				
	Yowaka River													4
	Wallagaraugh River													4
	Nyerimilang River		3	1										
West of Bass Strait	Curdies River			3					1	3	1			
	Hopkins River			5		1				1		1		
	Glenelg River			7						1				
Western Australia	Wilson Inlet			1				3						
	Swan River	2	1		1									

The Bayesian method of phylogenetic inference resulted in three groups of haplotypes (Figure 2). Well supported (posterior probabilities, pp. 1.0) group 3 comprises a single haplotype (hapK) that was found exclusively in the Yowaka and Wallagaraugh Rivers in southern New South Wales and formed a sister group with *F. macrodon* (pp 0.91). The sister group relationship of the group plus *F. macrodon* with *F. miamiensis* was also well supported (pp. 0.9). Group 2 (found in all other populations; Table 1) comprised seven haplotypes and was weakly supported (pp. 0.86). Group 2 was found in the same locations as Group 3, except in the Swan River in Western Australia), comprised six haplotypes and was well supported (pp. 0.97). The sister group relationship of groups 1 and 2 was well supported (pp. 0.97), but the relationships among the three groups remained ambiguous as the groups 3 and (1 plus 2) formed an unresolved polytomy.

Divergence among haplotype groups was substantial, as shown in Table 2, with the minimum divergence observed between group 1 and group 2 haplotypes at 19.2%. Divergence between group 3 and *F. macrodon,* for which a sister relationship was suggested, was also at the lower end of the divergence levels observed; at 25.7%. The remainder of pair-wise comparisons of divergence between haplotypes groups and species were high, in excess of 29%.

Analysis of patterns in ISSR profiles

Across the two primers, seventy one iSSR loci were identified with a band scored in at least one individual. Two samples which showed no bands for one or both of the primers were excluded as poor samples. Unique banding patterns across 29 samples were found for all loci and none of the samples had the same overall profile of banding across loci. No

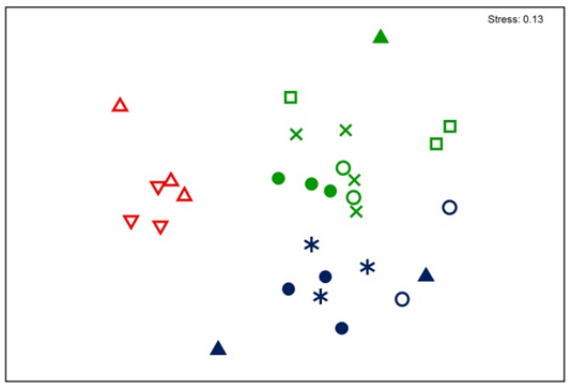

Figure 3. Multidimensional scaling plot of (Jaccard) dissimilarity in iSSR profiles among worms sub-sampled from the three genetic groups resolved during mtDNA sequencing. Collection location of samples is indicated by symbol type (Cooks River, hollow circles; Yowaka River, hollow upwards triangles; Wallagaraugh River, hollow downwards triangles; Nyerimilang River, hollow squares; Curdies River, solid circles; Hopkins River, solid upwards triangles; Wilson Inlet, star; Swan River, cross) while mtDNA group is indicated by symbol colour (1, green; 2, blue; 3, red).

loci were scored for a band in every individual and there were no diagnostic loci where all individuals in one group were scored with a band but all individuals in the other groups were not. A number of loci were nearly diagnostic, however, with most individuals in a group being positive for a band, in contrast to no (or just one) individuals with a band in the other groups*;* a full summary of banding profiles is given in the Supplementary material Table S2 and Table S3.

There were clear differences in the average similarity of ISSR profiles among sequenced based groups (Figure 3). PERMANOVA testing for any

Table 2. Pairwise distances among *Cyt B* sequences of *Ficopomatus* taxa. Mean corrected intergroup distance (Tamura-Nei model, above diagonal) and uncorrected (below diagonal) inter-clade and intra-clade (along diagonal). Higher values of corrected distances were due to nucleotide saturation at the third codon positon. *Hydroides trivesiculosa* was included in analyses as an outgroup.

	GROUP 1	GROUP 2	GROUP 3	*F. macrodon*	*F. miamiensis*	*H. trivesiculosa*
GROUP 1	0.007	0.231	0.390	0.462	0.536	0.633
GROUP 2	0.192	0.097	0.512	0.471	0.568	0.577
GROUP 3	0.294	0.356	0.003	0.327	0.540	0.520
F. macrodon	0.324	0.336	0.257	-	0.502	0.518
F. miamiensis	0.372	0.387	0.361	0.351	-	0.518
H. trivesiculosa	0.403	0.389	0.359	0.363	0.429	-

differences among groups was significant ($F = 4.901$, $p\ (perm)_{2, 26} = 0.001$) and subsequent pairwise testing detected differences between groups for each of the three pairwise comparisons ($p \leq 0.002$ for all comparisons). Analysis with PERMDISP failed to detect a difference in multivariate dispersion among the three groups ($F = 2.64$, $p\ (perm)_{2, 26} = 0.187$).

Discussion

Combined, our results did not show east-west phylogeographic patterns expected for populations of native *F. enigmaticus*, although neither do they rule out an Australian origin. It is clear that the phylogeography of *F. enigmaticus* in Australia is more complex than we expected. Instead, our results suggested the presence of three distinct genetic groups across southern Australia within the worms collected as *F. enigmaticus*. Mitochondrial (*Cyt B*) sequence data resolved three groups, including two widespread groups (1, 2) found together on both sides of the continent and a distinct group (3) found only in two creeks in the south east of Australia. When ISSR profiles were measured, PERMANOVA analysis found clear differences aligned with the sequence based groups, which appeared to be consistent across populations and biogeographic regions. Given the independent nature of the genetic markers (mitochondrial sequences, nuclear ISSR), we conclude that each sequence-based group is likely a separate species, albeit at this stage the identity of each of these is difficult to discern.

Ficopomatus enigmaticus belongs to a distinct clade of serpulid polychaetes (see Kupriyanova et al. 2009) that includes six species of the genus *Ficopomatus* Southern, 1921, all found in brackish water locations worldwide, plus the only known freshwater-obligate cave-inhabiting serpulid, *Marifugia cavatica* Absolon and Hrabě, 1930. Until now, the only other species of *Ficopomatus* recorded in Australia (*F. uschakovi* (Pillai, 1960)) had been recorded from locations ~ 500 km further north of our most northerly site in Sydney (ten Hove and Weerdenburg (1978: 110) and is generally considered a tropical rather than temperate species (ten Hove and Weerdenburg 1978). Consequently, we had assumed that all the worms we had collected across southern Australia were *F. enigmaticus*. Having exhausted our initial samples for the genetic work, we have subsequently collected some additional worms for more detailed morphological examination from two locations we previously sampled genetically – Yowaka River in southern NSW (group 3; collected November 2012) and Hopkins River in south west Victoria (groups 1, 2; January 2012), adding to material already held in the Australian Museum.

We have been unable to detect any obvious morphological differences among worms in the subsequent Hopkins River sample that might indicate two species living in sympatry, although we cannot guarantee the subsequent sample contained both group 1 and 2 worms; albeit both groups were common during our genetic sampling and we have no reason to suspect that our second sample should not contain both. Thus, similar to a marine intertidal serpulid cryptic pair from southern Australia (*Galeolaria caespitosa* and *G. gemineoa*; Halt et al. 2009) that have been differentiated by molecular genetic differences alone, we may have detected truly cryptic species within *F. enigmaticus*. Although Halt et al. (2009) were willing to name morphologically cryptic sister *Galeolaria* species on the basis of genetic sequence differences, in that case there was also other reasonable evidence that the two were isolated, geographically and reproductively; i.e., the two species were found either side of the Bass Strait biogeographic boundary, which also correlated with previous patterns of breeding (gametic) incompatibility shown between populations of worms across that boundary (Styan et al. 2008). At this stage, we believe further work is needed before describing the two groups (1, 2) of worms we found within *F. enigmaticus* as separate species, particularly because

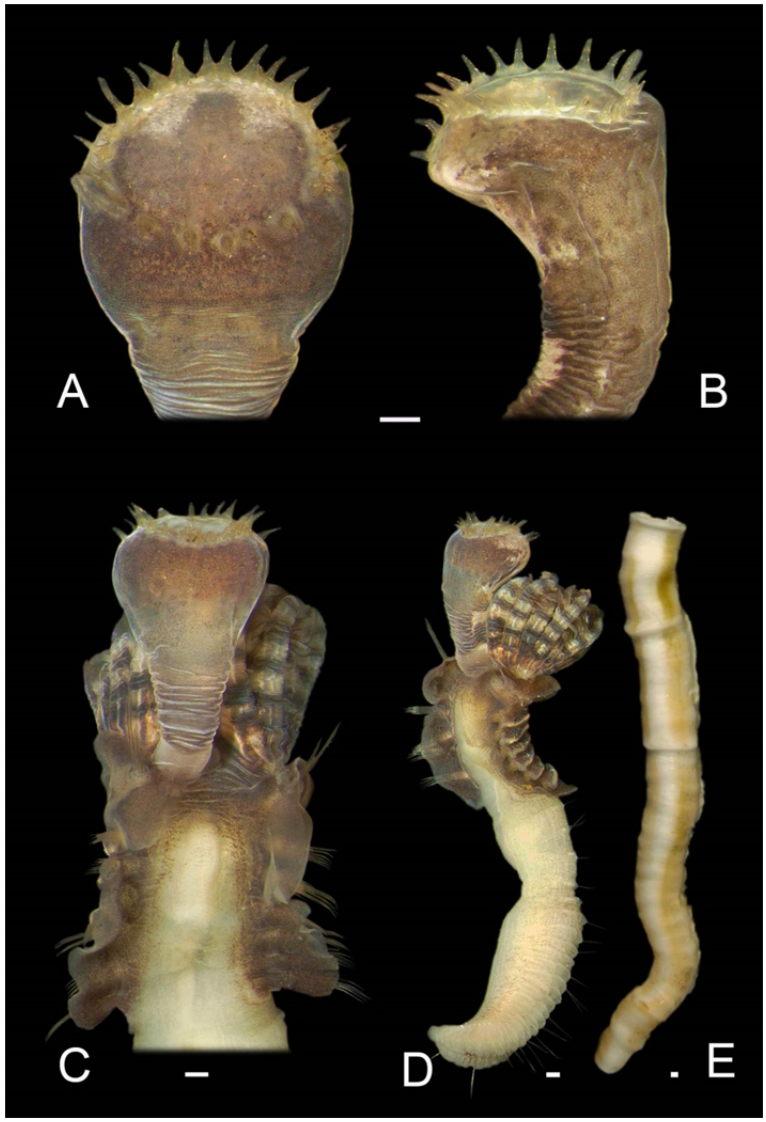

Figure 4. Photographs of live *Ficopomatus* cf. *uschakovi* from Pambula creek (Yowaka River) in southern NSW, AM W.45641. A, B – views of opercula, C – ventral view of the anterior end, D – ventro-lateral view of an entire worm removed from its tube, E – tube. Scale bar = 0.1 mm. Photo E. Wong.

the two groups were found in close sympatry (literally within the same clumps) and in almost equal (and large) numbers in many places, suggesting they are often found close enough to each other in nature to potentially interbreed via broadcast spawning.

Unfortunately, for *F. enigmaticus sensu lato* we do not have any direct measure of the level, if any, of interbreeding that might be occurring between groups 1 and 2 in sympatric populations because heterozygosity cannot be identified with mitochondrial sequences or dominant markers like iSSRs. While differences detectable in similarities in iSSR profiles for groups based on the *Cyt B* clades confirms there was not complete random mating between the clades, the PERMANOVA results alone cannot rule out (or quantify) some level of genetic introgression. If there had been hybrids between groups 1 and 2 in our samples, however, we might have expected to then see increases in the multivariate dispersion of similarities in iSSR profiles within clades 1 and/or 2 relative to group 3; i.e., the presence of hybrids would be expected to increase the range of similarities of iSSR profiles within a group, relative to the geographically separate and distinct group 3, which we assume would not contain hybrids. Given no such differences were detected using PERMDISP analysis, this may be indirect evidence for the absence of hybrids in our samples. Nonetheless, we believe a more detailed morphological study and/or other genetic evidence is needed to assess just how

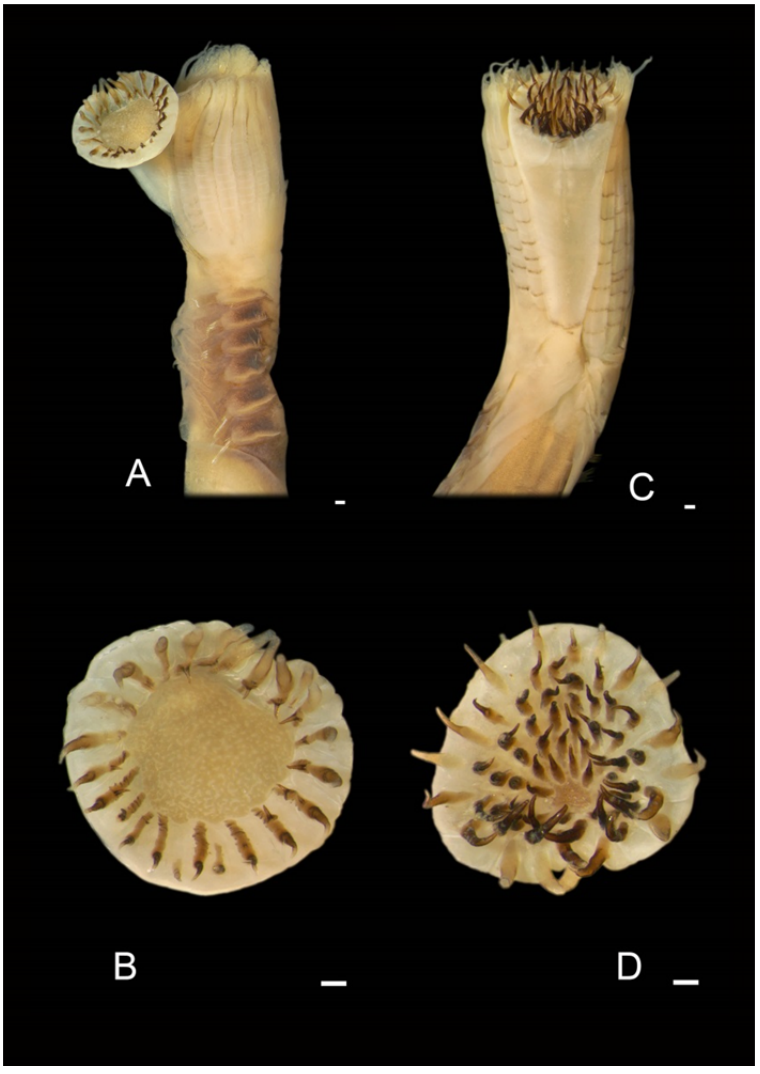

Figure 5. Photographs of *Ficopomatus enigmaticus* from Paramatta River between Homebush Bridge and Ryde Bridge, NSW, AM W.26262. A – lateral view of the anterior end; B –ventral view of the anterior end; C, D – top views of opercula. Scale bar = 0.1 mm. Photo E. Wong.

reproductively isolated these sympatric groups are, before specific status is formally described to one (or more) of groups 1 and 2.

Moreover, even with further evidence that groups 1 and 2 are sufficiently separated genetically to justify describing them as distinct species, for example, with measures of gametic incompatibility that might occur between taxa (c.f. Styan et al. 2008), a key issue would then be determining which genetic group (if any) was represented by the first, formal description of *F. enigmaticus* from France and which group should be described as a new species. Without characteristic morphological traits to compare with the syntype description (ten Hove and Weerdenburg 1978), assigning either genetic group as new would be arbitrary. Certainly, if future genetic surveys of contemporary populations in France found only one group but not the other, then that might suggest which genetic group the syntype was likely to align, but without directly sequencing a syntype there will always be some level of inference required to assign which genetic group is new. As yet though, our morphological examinations have been preliminary and we hope that future more detailed morphological work across multiple populations linked with genetic characterisation of individual samples might reveal morphological characteristics that could be used to help resolve the specific status of *F. enigmaticus sensu lato* within Australia.

We did, however, subsequently find that (group 3) worms from Yowaka River do look different from *F. enigmaticus* (Hopkins River sample) and instead more closely resembled descriptions of *F. uschakovi* (see Pillai 1960; ten Hove and Weerdenburg 1978

and Figure 4). If the group 3 worms are *F.* cf. *uschakovi*, this would be a significant (~ 1000 km south) range extension, although it is unclear whether they might have actually been living undetected in south eastern NSW and Victoria for some time. *Ficopomatus uschakovi* originally described from Sri Lanka is reported as naturally widely distributed across the tropical Indo-West Pacific, including Northern Australia (ten Hove and Weerdenburg 1978). Like *F. enigmaticus*, *F. uschakovi* has apparently invaded a number of places around the world—having been recorded in the Eastern Atlantic, in the Gulf of Guinea (Hartmann-Schröder 1971), the Western Atlantic, in Brazil (de Assis et al. 2008), in the Mexican Pacific (Bastida-Zavala and García-Madrigal 2012), in Venezuela (Liñero-Arana and Díaz-Díaz 2012) and the Colombian Caribbean (Arteaga-Flórez et al. 2014).

Ten Hove and Weerdenburg (1978) have noted that *F. uschakovi* has been commonly misidentified as *F. enigmaticus* (see Figure 5). Morphologically, *F. uschakovi* is characterized by a spherical operculum with transparent spines directed outwards in a radial arrangement (as opposed to dark spines curved inwards in *F. enigmaticus*), and dorsal fusion of the thoracic membranes (as opposed to free thoracic membranes in *F. enigmaticus*). Opercula of the worms from the Yowaka River are typical of *F. uschakovi* (Figure 4A, B), but have free thoracic membranes found in all *Ficopomatus* spp., except for *F. uschakovi.* Consequently, our suspicion is that the group 3 worms may not be *F. uschakovi* at all, but rather a closely related, undescribed species. Unfortunately, lack of genetic data from *F. uschakovi sensu stricto* does not allow comparisons and a wide genetic survey across the tropical (putative native) ranges of *F. uschakovi* in Australia and Asia, as well as invasive populations elsewhere, are needed to resolve the invasion status of the temperate Australian populations of *F.* cf. *uschakovi*. The clade 3 grouped with *F. macrodon* Southern, 1921, the species that easily distinguishable morphologically from *F. uschakovi* and *F. enigmaticus* by the operculum with flat horny endplate lacking any spines.

At the start of this study we had planned to look at phylogeographic patterns within a single species (*F. enigmaticus*) across Australia and sampled what we thought were sufficient numbers of worms from enough widespread populations to do this. The presence of three (probable species level) genetic groups consequently meant that our sample sizes were far too small to say anything about connectedness among populations within any of the groups (species). Nonetheless, our data do indicate that the phylogeography of the genus *Ficopomatus* is complex and potentially paraphyletic within Australia, needing more work and additional genetic markers to resolve. Moreover, we suspect that a future global phylogeographic study of *Ficopomatus* might reveal other undescribed species that may also be important for unravelling the origin of worms in different locations. Given the presence of multiple species within nominal *F. enigmaticus* in southern Australia, the next most important information for understanding where populations may have originated is determining whether non-Australian worms match with any of the (possibly three) species of *Ficopomatus* in southern Australia. Obviously, France, where the species was described from would be a high priority starting point. Similarly, it will be important to determine whether groups 1 and 2 occur in sympatry or allopatry across other areas where *F. engimaticus* might also be native (e.g., South Africa, South America, and Europe). After that, more detailed genetic analysis with other markers and larger sample sizes will be needed to unravel translocation pathways within/outside of natural ranges (e.g., Geller et al. 2010; Perez-Portela et al. 2013; Lejeusne et al. 2014). Thus, further studies are still needed from across Australia and overseas to untangle the unexpectedly confusing phylogeography of species within the *Ficopomatus enigmaticus* complex, but this study has at least provided an understanding of the complexity of the problem.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Locations of Australian collection sites

Table S2. Banding profiles for iSSR primer 818 (*Ficopomatus enigmaticus sensu lato*)

Table S3. Banding profiles for iSSR primer 827 (*Ficopomatus enigmaticus sensu lato*)

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