**Distribution and population genetics of the threatened freshwater crayfish genus
Tenuibranchiurus (Decapoda: Parastacidae)**

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Abstract. Very high rates of extinction are recorded in freshwater ecosystems, with coastally distributed species threatened by urban development, pollution and climate change. One example, the world's second smallest freshwater crayfish (genus *Tenuibranchiurus*), inhabits coastal swamps in central-eastern Australia. Although only one species is described (*Tenuibranchiurus glypticus*), it was expected that populations isolated through habitat fragmentation would be highly divergent. The aims of this study were to determine if: populations of *Tenuibranchiurus* are genetically distinct, and if ancient divergence, as indicated in other species in the region, was evident. *Tenuibranchiurus* were collected at seven sites, extending the known geographical distribution ~260 km south to Woolli, NSW. Analysis of two mitochondrial DNA gene regions indicated two highly divergent clades, with numerous additional sub-clades. Both clades and sub-clades were strongly congruent with geographical location, and were estimated to have diverged from each other during the Miocene/Pliocene era. Little sharing of haplotypes between sub-populations was evident, indicating negligible gene flow, and genetic differentiation between sub-clades possibly indicates distinct species. The coastal distribution of *Tenuibranchiurus*, severe habitat fragmentation and clear differences between sub-clades suggests they should be recognised as evolutionarily significant units, and be treated as such if conservation and management initiatives are warranted.

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Introduction

With extinctions occurring in freshwater ecosystems faster than any other habitat (Ricciardi and Rasmussen 1999; Loh 2002; Jenkins 2003), it is critical to understand current patterns of diversity in our rivers and streams. High levels of cryptic diversity have been found in many freshwater species (Baker *et al.* 2003; Baker *et al.* 2004; Cook *et al.* 2006) and in many cases, these cryptic species appear to have diverged during the Miocene or Pliocene. Despite similar morphologies, they often have different distributions from one another (Chenoweth and Hughes 2003; Cook *et al.* 2006; Page and Hughes 2007). It is vital to determine the levels of cryptic diversity and the patterns of distribution for freshwater species to conserve and maintain biodiversity. If these factors are ignored, it is likely that management plans will be ineffective and possibly result in loss of species (Margules and Pressey 2000; Cook *et al.* 2008a).

One practice for maintaining the evolutionary heritage of populations is through the identification of evolutionarily significant units (ESUs). There are many definitions as to what constitutes an ESU; however, Moritz (1994) attempted an operational definition, specifying that ESUs should be “reciprocally monophyletic for mtDNA and show significant divergence of allele frequencies at nuclear loci”. Based on this definition, many ESUs have been identified within previously described crayfish species, including *Cherax tenuimanus* (Smith) from Western Australia (Nguyen *et al.* 2002), and *Austropotamobius pallipes* (Lereboullet) from France (Gouin *et al.* 2006), Italy (Fratini *et al.* 2005) and the Iberian Peninsula (Dieguez-Urbeondo *et al.* 2008).

In Australia, freshwater crayfish are of particular interest because a number of species inhabit small coastal streams throughout Queensland (Qld) and New South Wales (NSW). Much of their habitat is highly fragmented, often separated by extensive areas of unsuitable or degraded habitat (mostly urban development). Many of these species are habitat specialists and are often geographically isolated from one another, a situation likely to lead to genetic divergence and possibly speciation over evolutionary time. Studies on a number of freshwater species in the region have shown evidence of high levels of genetic divergence among some populations as well as phylogeographic breaks along the NSW and Qld border, including *Macrobrachium australiense* Holthius (Carini and Hughes 2004; Sharma and Hughes 2009), *Cherax*

65 *dispar* Riek (Bentley *et al.* 2010), *Rhadinocentrus ornatus* Regan (Page *et al.* 2004)
and *Nannoperca oxleyana* Whitley (Hughes *et al.* 1999; Knight *et al.* 2009). Because
previous studies demonstrate that these organisms living in coastal habitats exhibit
high levels of genetic divergence, it is likely that *Tenuibranchiurus*, a genus of
freshwater crayfish confined to the same coastal habitat (i.e. a similar distribution),
70 also consists of a series of genetically divergent populations.

Tenuibranchiurus (Decapoda: Parastacidae) is a little known freshwater crayfish
genus found along the central eastern coast of Australia (Riek 1969). It is regarded as
the second smallest freshwater crayfish worldwide, reaching a maximum total length
75 of only 25 mm (Riek 1969; Crandall 2002). Studies on *Tenuibranchiurus* to date
have concentrated mainly on taxonomic and phylogenetic relationships with other
Australian crayfish genera, rendering the genus poorly understood at a basic level.
For example, there has been no concentrated efforts to determine the actual
distribution of the genus, and almost no information is available on its basic biology
and ecology, with only two studies on the general behaviour of *Tenuibranchiurus*
80 (Harding and Williamson 2003; Harding and Williamson 2004).

There is currently one species described in this genus, *Tenuibranchiurus glypticus*
Riek, but the presence of two additional species has been suggested on the basis of
85 morphological differences (Horwitz 1995). As a result of long-term historical
geographic isolation, sub-populations of *Tenuibranchiurus* may have undergone
genetic divergence, possibly resulting in genetically distinct species within this genus.
Currently, this genus has no conservation status assigned to it, and is only protected
where it occurs in National Parks and other conservation areas. Appreciable areas of
90 its habitat have been cleared for development such as housing, business and
infrastructure (Joyce 2006). Therefore, the genus may require protection due to
significant and continuing habitat loss restricting and reducing its distribution and
eliminating isolated sub-populations. However, *Tenuibranchiurus* may also warrant
conservation efforts on the basis of geographically isolated sub-populations qualifying
95 as ESUs (as defined by Moritz 1994). Therefore, the aims of this study were to
investigate (1) if sub-populations of *Tenuibranchiurus* are genetically isolated from
one another; and (2) if there is evidence of ancient divergence as has been
demonstrated for other aquatic species occupying similar coastal habitat in this region.

Materials and methods

Study species

Tenuibranchiurus is an unremarkable grey-brown crayfish that is cryptic in habit (Riek 1951). The genus was first described from two locations, Mt Gravatt and Caloundra, Qld, by Riek (1951). Since then, the genus has been reported from a total of five locations within Qld; Bells Creek (Crandall *et al.* 1999; Schultz *et al.* 2007; Schultz *et al.* 2009), Eumundi, Mooloolaba, Kinkuna National Park (Schultz *et al.* 2007) and Bribie Island (Harding and Williamson 2003; Schultz *et al.* 2009), and also in ‘far north-eastern’ NSW (Horwitz 1995) (Fig. 1B).

Tenuibranchiurus inhabits coastal wallum/*Melaleuca* swamps where they are thought to construct deep burrows in the soil (Riek 1951). *Tenuibranchiurus* can be readily distinguished from most other crayfish in the region by the position of the chelae, which are orientated vertically instead of horizontally (Riek 1969). They are quite similar in morphology to the genus *Engaeus* (from Tasmania and Victoria), but differ in branchial and abdominal structure (Riek 1951; Riek 1969). Although *Tenuibranchiurus* resemble *Engaeus* in form, they are genetically more closely related to the genus *Geocharax* (from Tasmania and Victoria), being placed “sister” to these in a study undertaken by Schultz *et al.* (2007).

Sampling methods

Sampling was carried out during the day from January to May 2008. Sites of potential habitat for *Tenuibranchiurus* were identified from digital vegetation maps detailing coastal *Melaleuca* swamp distributions. Additional sites that appeared to be suitable habitat were also identified opportunistically while in the field. *Tenuibranchiurus* were collected from swamps by sweep netting, pumping out burrows with a bait-pump and baited traps. Where the bait-pump could not be used efficiently (e.g. tree roots obstructing pump), hand excavation of the burrows was undertaken. Where possible, a minimum of five individuals were collected from each site as this sample size has been considered in other studies to be sufficient to provide a robust genetic analysis to identify deep levels of genetic divergence (e.g. Ponniah and Hughes 2004). Live specimens were stored separately on ice in the field, frozen at -20°C on return to the laboratory, and preserved separately in 70% ethanol. Fifty-

one *Tenuibranchiurus* were collected over seven field sites and retained for genetic analysis.

135 *DNA extraction, amplification and sequencing*

The DNA from each specimen was extracted using a variation of the CTAB/phenol-chloroform extraction protocol (Doyle and Doyle 1987). Two mitochondrial regions were amplified; cytochrome oxidase subunit 1 (COI) using the primers CRCOI-F (5'-CWACMAAYCATAAAGAYATTGG-3') and CRCOI-R (5'-GCRGANGTRAARTARGCTCG-3') (Cook *et al.* 2008b), and 16S using the primers 16S-ar (5'-CGCCTGTTTATCAAAAACAT-3') and 16S-br (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.* 1991).

The PCR reactions for both COI and 16S used the following; 2.0 µL DNA extract, 0.4 µL forward primer, 0.4 µL reverse primer, 0.3 µL dNTP, 0.63 µL MgCl₂ (50 mM), 2.50 µL buffer (5×), 0.08 µL Bioline Mango *Taq* (5×) and 6.19 µL ddH₂O. For all analyses, one positive and one negative control were included, with PCR reactions performed on a GeneAmp PCR System 2700 (Applied Biosystems, www.appliedbiosystems.com). The PCR program used for COI samples consisted of the following conditions: 94°C for 5 min; 40 cycles of: 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C; then 7 min at 72°C with the reaction stored at 4°C until further analysis. The program used to amplify 16S samples was: 94°C for 10 min; 40 cycles of: 45 sec at 94°C, 45 sec at 52°C, 1.5 min at 72°C; then 7 min at 72°C with the reaction stored at 4°C until further analysis.

155 An enzymatic purification procedure was undertaken using EXO SAP (Fermentas, www.fermentas.com) on successfully amplified samples. Purifications were performed in 6.25-µL reaction volumes containing the following: 0.25 µL Exonuclease I, 1.0 µL Shrimp Alkaline Phosphate and 5.0 µL amplified PCR product. The resulting solution was incubated at 37°C for 35 min, heated at 80°C for 20 min, held at 15°C, then stored at 4°C until further analysis. Sequencing reactions contained 1.0 µL purified PCR product, 5.5 µL ddH₂O, 5.0 µL Terminator Mix (Applied Biosystems, www.appliedbiosystems.com), 2.0 µL 5×Terminator Mix Buffer (Applied Biosystems, www.appliedbiosystems.com) and 1.0 µL of the forward primer (CRCOI-F/16S-ar). Reactions were performed under the following conditions:

initial hold of 96°C for 1 min; 30 cycles of: 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min; then a final hold at 4°C. Samples were then cleaned and subsequently sequenced on an automated sequencing machine (Applied Biosystems 3130, www.appliedbiosystems.com). Sequences were edited using Sequencher 4.1.2 (GeneCodes 2000) and aligned using MAFFT version 5.3 (Kato *et al.* 2005). A 644-bp fragment was used in COI analyses and a 449-bp fragment for 16S analyses.

COI and 16S phylogenetic analyses

A total of 51 *Tenuibranchiurus* collected during this study were sequenced for the COI gene fragment, with a subset of 14 sequenced for the 16S fragment to examine deeper phylogenetic relationships. Additional sequences obtained from GenBank (see Accessory Publication) were also included in both COI and 16S analyses. For all analyses, *Gramastacus* sp. sequences were used as an outgroup as this genus (with *Engaewa* and *Geocharax*) has been demonstrated as forming a monophyletic clade with *Tenuibranchiurus* to the “exclusion of all other parastacid genera” (Schultz *et al.* 2009).

To examine the relationship between *Tenuibranchiurus* haplotypes, a best-fit model of evolution was first selected for both the COI and 16S data sets (COI=HKY+I+G model; 16S=HKY+G model) using the program jModeltest v. 0.0.1 (Posada 2008). For each data set, three methods were used; neighbour-joining (NJ), maximum likelihood (ML) and maximum parsimony (MP). Both the NJ and MP trees were created using the program PAUP* (Swofford 2003) (1000 bootstrap replicates; full heuristic search), while the ML tree was created using the program RAxML v. 7.0.0 (Stamatakis 2006) (1000 bootstrap replicates). Networks showing relationships among haplotypes were also created for the COI data set, using the program TCS v. 1.21 (Clements *et al.* 2000). Networks were not created for the 16S data as only 25 sequences were available for analysis (see Accessory Publication).

Time of divergence

The time of divergence between identified clades and sub-clades was estimated using a molecular clock approach using MEGA (Tamura *et al.* 2007). Using a molecular clock approach was considered appropriate as tests performed on both unconstrained and constrained trees showed that they were not significantly different ($p=0.828$).

200 The net sequence divergence was calculated (including $\pm$ S.E. to correct for within-clade polymorphisms) and three estimated divergence rates were used for both COI and 16S; COI=2.0% (Wares and Cunningham 2001), 1.7% (Schubart *et al.* 1998) and 1.4% (Morrison *et al.* 2004; Page and Hughes 2007); 16S=0.9% (Sturmbauer *et al.* 1996; Schultz *et al.* 2009), 0.65% (Schubart *et al.* 1998) and 0.53% (Stillman and 205 Reeb 2001; Schultz *et al.* 2009).

Analysis of population structure

All further statistical analyses were only performed on the COI data as the 16S sample sizes were too low for robust analyses. Two tests of neutrality were carried out 210 (Tajima's *D* (Tajima 1989) and Fu's *F_s* (Fu and Li 1993)) using Arlequin 3.1 (Excoffier *et al.* 2005). These tests were used to detect any recent demographic changes (e.g. recent bottlenecks or population expansions) or non-neutral evolution of the COI fragment.

215 As there was no real logic for dividing the sample sites into regions or groups, analysis of molecular variance (AMOVA) was not performed. Instead, pairwise Φ_{ST} (genetic structure based on haplotype frequency and genetic divergence) were calculated using Arlequin to determine the level of genetic differentiation between pairs of sites. Where sites were represented by fewer than 5 individuals, they were 220 either pooled (i.e. MA with TCB, GC1 with GC2, BNP1 with BNP 2) to provide a robust analysis, or were removed (i.e. BI). A Mantel test with 1000 permutations was also run in Arlequin to identify any relationships between genetic and geographic distance between *Tenuibranchiurus* sites, which would be expected if the dispersal distance of individuals is less than the area of the study and the sub-populations have 225 been in their habitat for long enough to have reached equilibrium between migration, mutation and genetic drift (Slatkin 1993).

Results

Field summary

In addition to historical locations where *Tenuibranchiurus* had previously been recorded (see Accessory Publication), seven additional sub-populations were identified (from 31 field sites sampled) with a number of these being outside the previously reported range of *Tenuibranchiurus*, extending its known distribution. Although all historical sites were visited for sampling, no *Tenuibranchiurus* were found, either due to habitat loss or lack of water due to drought conditions. In general, site characteristics where crayfish were collected were typical of those previously described in the literature (i.e. coastal wallum/*Melaleuca* swamp); however, two sites were notably different on the basis that the first (LakeH) was a natural drainage channel in heathland with no *Melaleuca* present and the second (BNP2) was a deep stream channel, with slow-flowing water, with *Melaleuca* on the stream banks.

Phylogenetic analysis

Fifty-one *Tenuibranchiurus* collected during this study were sequenced for the COI gene fragment, with an additional six *Tenuibranchiurus* samples obtained from Bentley (2007) and five specimens of *Gramastacus* sp. which were used as outgroups. Within *Tenuibranchiurus* COI sequences, 175 of 644 bases were variable with 20, 4 and 151 1st, 2nd, and 3rd codon position changes, respectively, and a total of 13 amino acid changes. A total of 154 transitions and 51 transversions were observed across all nucleotide sites, with 158 of these sites parsimony informative. No stop codons were observed in any of the sequences. From the 57 individuals, a total of 28 haplotypes were identified. All of the phylogenetic trees (NJ, ML and MP) inferred from the 28 *Tenuibranchiurus* haplotypes (with outgroups) suggest the presence of two highly divergent clades (bootstrap values 100%) with two and three sub-clades, respectively, within each (bootstrap values $\geq 95\%$) (Fig. 1A). Clade 1 consisted of haplotypes from the Qld region, while Clade 2 were those from NSW.

From Sub-clades 1 through 5, three to four representative haplotypes were sequenced for the 16S gene fragment. Additional 16S sequences were obtained from GenBank and Bentley (2007) (see Accessory Publication), and four *Gramastacus* sp. samples were used as 16S outgroups. Within *Tenuibranchiurus* 16S sequences, 94 of 449

bases were variable. A total of 85 transitions and 29 transversions were observed across all nucleotide sites. The phylogenetic trees inferred from the 22 *Tenuibranchiurus* 16S haplotypes showed the same two divergent clades, but the sub-clades were not as clear. However, the same groupings were noted, with Clade 1 comprised of haplotypes found only in Qld and Clade 2 of those from NSW (Fig. 1C).

Haplotype network analysis

Five COI networks were created (Fig. 1B) using the program TCS. However, these were unable to be joined despite using a cut-off level of 90%. The haplotypes displayed concordance with geographical location of sub-populations (Fig. 1B). Sub-clade 1 consisted of those individuals found at sites GC1 and GC2 (Gold Coast, Qld), Sub-clade 2 from MA and TCB (Maryborough and Tin Can Bay, Qld), Sub-clade 3 from LakeH, BNP1 and BNP2 (Lake Hiawatha and Broadwater National Park, NSW), Sub-clade 4 from LH (Lennox Heads, NSW), and Sub-clade 5 from BI2 (Bribie Island, Qld).

Time of divergence

Based on the COI gene, it is estimated that divergences between each of the five *Tenuibranchiurus* sub-clades occurred during the Miocene (5 – 23mya) and Pliocene era (1.8 – 5mya) (Table 1). The most recent split was between Sub-clades 2 and 5 (2.5 – 3.5mya), with the earliest between Sub-clades 1 and 3 (11.1 – 15.9mya). As the 16S gene fragment was used to examine deeper phylogenetic relationships, the time of divergence was only estimated between the two major clades (Clades 1 and 2). The time of divergence was placed 16.1 – 27.4mya, which was slightly older than the estimate based on COI (8.0 – 11.4mya).

Analysis of population structure

Tajima's *D* test of neutrality was non-significant for all sub-clades, while Fu's *F_s* was only significant for Sub-clade 1 ($p < 0.02$) (Table 2). All pairwise Φ_{ST} comparisons were significant ($p < 0.001$), with values 0.77 or greater indicating very little or no gene flow between sites (Table 3). The Mantel test showed a weak but significant relationship between the Φ_{ST} and geographical distance ($p = 0.044$).

Discussion

This study extends the distribution of *Tenuibranchiurus* appreciably (~260 km), with this genus not previously recorded on the Gold Coast in Qld or as far south as Wooli, NSW. The total known range now extends approximately 600 km north-south, and it is possible that the distribution extends further north and south of these present limits. Also, LakeH and BNP2 were markedly different from the other sites sampled, indicating that habitat previously thought unsuitable for *Tenuibranchiurus* (i.e. Riek 1951) may in fact be suitable for this genus.

Genetic isolation of Tenuibranchiurus sub-populations

The results of the pairwise Φ_{ST} analysis suggest high levels of genetic diversity and very little or no gene flow between *Tenuibranchiurus* sub-populations. Similar results have also been reported for other organisms that have experienced some form of population separation and are found in similar habitats to *Tenuibranchiurus*. For example, very low levels of dispersal between sub-populations of the Oxleyan pygmy perch (*Nannoperca oxleyana*) have been reported, with Φ_{ST} values between 0.25 and 0.91 (Hughes *et al.* 1999). Bentley *et al.* (2010) also examined levels of gene flow within four clades of *Cherax dispar* and found very little gene flow between sub-populations (Φ_{ST} between 0.33 and 0.83).

All but one of the Tajima's *D* and Fu's *F_s* tests of neutrality were non-significant ($F_s = -4.10$, $p < 0.02$). Although this value is significant, it is small compared to values found in other studies (up to -72.7) (Mills *et al.* 2008). Taken together, the results of these tests suggest there is no evidence of recent bottlenecks or population expansions within the five sub-clades tested. There is very little sharing of haplotypes between sub-populations, indicating that at least female dispersal rates are very low, which is not surprising due to the large geographic distances that separate sub-populations. As mtDNA was analysed for this study, no conclusions can be made as to the movements of the male individuals, as mtDNA is maternally inherited (Hartl and Clark 2007). Nevertheless, there is no reason to suspect male-biased dispersal in these crayfish.

Time of divergence

Both the COI and 16S trees show a clear separation between Clade 1 and Clade 2. This is interesting, as Clade 1 is comprised of samples from Qld and Clade 2 of

samples from NSW. The separation is quite large, almost as large as the separation between the *Tenuibranchiurus* samples and the outgroup (*Gramastacus* sp.). This extensive divergence may be due to a long-term barrier. It is possible that the mountain ranges that occur on the border of Qld and NSW have restricted the movement of individuals between northern and southern regions. The analysis of sequence variation in the COI gene fragment also shows clear differences between the sub-clades identified. Similar relationships between sub-clades were also evident for the 16S gene. Also, the 16S phylogenetic tree shows that the samples obtained from the *T. glypticus* type locality (Sub-clade 8; Bells Creek, Caloundra) are distinct from other groupings evident within Clade 1.

Both the COI and 16S data sets suggest that *Tenuibranchiurus* sub-clades diverged during the Miocene/Pliocene era. There has been one other study that has suggested that the geological age of *Tenuibranchiurus* is only as old as the Holocene era (i.e. 12000ya – present) based on morphological characteristics (Rode and Babcock 2003); however, the estimates obtained from this study clearly indicate that *Tenuibranchiurus* is far older, and the analyses show that the oldest divergence occurs between Qld and NSW. Another genus of crayfish, *Euastacus*, which occupies high altitude areas in eastern Australia, has been shown to have diverged more recently, during the Pliocene era (Ponniah and Hughes 2004). This was surprising as there are several clearly morphologically recognised species within the genus *Euastacus*, in contrast to the one recognised *Tenuibranchiurus* species. It is possible that further investigation of the morphology and taxonomy of *Tenuibranchiurus* may indicate at least two distinct species within this genus (i.e. Clades 1 and 2) if not more. Other freshwater crustaceans with a coastal distribution in this region (e.g. freshwater shrimp; *Caridina indistincta* Calman) have been found to have similar divergence times to those found for *Tenuibranchiurus*. *Caridina indistincta* inhabits coastal streams within south-east Qld, Australia, and divergence times between cryptic species fall within the Miocene era (estimates from 6.45 – 11.3mya) (Chenoweth and Hughes 2003; Page and Hughes 2007). Similarly, identified lineages within the shrimp species *Paratya australiensis* Kemp collected from south-east Qld and NSW have also been found to have diverged during the late Miocene/Pliocene (3.86 – 11.32mya) (Cook *et al.* 2006).

Implications for management

The distinct separation between *Tenuibranchiurus* sub-clades is indicative of high genetic divergence. Also, each of the sub-clades consisted of haplotypes collected from only one or two nearby locations. The implication of this is that if sub-
365 populations within a sub-clade were to be lost, it is likely that the genetic information contained within that sub-population would not be found in any other. The large geographic distances between sub-populations and the high levels of genetic divergence between them also indicate that it is very unlikely that these areas could be naturally recolonised following any local extinction events.

370 The increasing occurrence of habitat destruction, degradation and fragmentation as a result of anthropogenic influences highlights this as being a very real threat to these isolated populations (e.g. Taylor *et al.* 2007). Although some crayfish are highly mobile organisms that can, given time, disperse over quite large distances (Lindqvist
375 and Huner 1999; Hughes and Hillyer 2003), the small size of this crayfish and low levels of habitat connectivity between sub-populations greatly reduce any possibility of gene flow or movement between them.

In light of the genetic differentiation between the sub-clades, it is appropriate that they
380 be considered ESUs for management and conservation purposes. Also, according to Moritz (1994), Lake Hiawatha from Sub-clade 3 should be considered a separate management unit, and should be treated as such if conservation and management plans are to be applied. It is likely that management plans and conservation efforts will be needed in the future, if not immediately, as there have already been population
385 reductions. For example, one of the type localities for *T. glypticus* has been developed for housing (sites in Mt Gravatt; Riek 1951) with the sub-populations previously located there most likely having been eliminated.

Due to the genetic divergence detected, it is recommended that a thorough
390 examination of the nuclear genetic makeup and morphology of individuals within each *Tenuibranchiurus* sub-clade be undertaken as it is possible that each of these represents a distinct species. Many studies have used genetic analysis to identify new species, and have gone on to use this information to recognise ESUs and make recommendations for conservation and management plans (Austin and Ryan 2002; de

395 Bruyn *et al.* 2004; Santos 2006). In light of this, examination of the morphology and
possible description of new species within the genus should be a priority area for
future research.

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Table 1. Estimated time of divergence between clades based on the CO1 gene. Mean net distance calculated using the Tamura-Nei model with a gamma distribution. Timing and error to the nearest one hundred thousand years. SC=sub-clade. Timing¹=0.020 per myr, ²=0.017 per myr, ³=0.014 per myr

Comparison	Mean net distance	Standard error	Timing ¹ (myrs)	Error (myrs)	Timing ² (myrs)	Error (myrs)	Timing ³ (myrs)	Error (myrs)
SC 1 vs. 2	0.084	0.014	4.2	0.7	4.9	0.8	6.0	1.0
SC 1 vs. 3	0.222	0.030	11.1	1.5	13.1	1.8	15.9	2.1
SC 1 vs. 4	0.208	0.028	10.4	1.4	12.2	1.6	14.9	2.0
SC 1 vs. 5	0.085	0.014	4.3	0.7	5.0	0.8	6.1	1.0
SC 2 vs. 3	0.217	0.029	10.9	1.5	12.8	1.7	15.5	2.1
SC 2 vs. 4	0.190	0.025	9.5	1.3	11.2	1.5	13.6	1.8
SC 2 vs. 5	0.049	0.009	2.5	0.5	2.9	0.5	3.5	0.6
SC 3 vs. 4	0.117	0.018	5.9	0.9	6.9	1.1	8.4	1.3
SC 3 vs. 5	0.218	0.030	10.9	1.5	12.8	1.8	15.6	2.1
SC 4 vs. 5	0.181	0.024	9.1	1.2	10.6	1.4	12.9	1.7
SC 1 vs. the rest	0.117	0.016	5.9	0.8	6.9	0.9	8.4	1.1
SC 2 vs. the rest	0.100	0.014	5.0	0.7	5.9	0.8	7.1	1.0
SC 3 vs. the rest	0.134	0.018	6.7	0.9	7.9	1.1	9.6	1.3
SC 4 vs. the rest	0.099	0.013	5.0	0.7	5.8	0.8	7.1	0.9
SC 5 vs. the rest	0.082	0.012	4.1	0.6	4.8	0.7	5.9	0.9

Table 2. Results of the neutrality tests for each sub-clade using CO1 data

Sub-clades	Tajima's <i>D</i>	<i>P</i> -value	Fu's <i>F_s</i>	<i>P</i> -value
1	-0.46217	0.326	-4.10171	0.004
2	0.95536	0.860	5.25412	0.981
3	-0.44615	0.372	1.58931	0.811
4	-1.56949	0.055	-1.53469	0.047
5	-0.81734	0.132	0.96133	0.609

Table 3. Pairwise Φ_{ST} using CO1 sequences. *n*=number of individuals used in analysis. All comparisons significant (*p*<0.001). Refer Fig. 1 and Accessory Publication for site localities.

Sites (<i>n</i>)	MA&TCB	GC1&2	LH	BNP1&2
MA&TCB (10)	-			
GC1&2 (12)	0.77	-		
LH (11)	0.87	0.98	-	
BNP1&2 (14)	0.89	0.98	0.98	-
LakeH (6)	0.85	0.98	0.99	0.77

Fig. 1. (B) Locations of *Tenuibranchiurus* collection sites in Queensland and New South Wales and their corresponding CO1 networks; diamonds = data from this study, circles = data from past studies, broken circles = type localities. Size of haplotype circles is proportional to haplotype frequency. Maximum likelihood bootstrap phylograms showing the relationship between (A) CO1 haplotypes and (C) 16S haplotypes, where colour shades correspond to sample location. Numbers on branches indicate ML bootstrap values, with parentheses the NJ and MP bootstrap values respectively. Refer to Accessory Publication for site localities (historical and this study).