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Keith Crandall / 13 Feb 2017

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A novel genus and cryptic species harboured within the monotypic freshwater crayfish genus *Tenuibranchiurus* (Decapoda: Parastacidae)

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Identifying species groups is an important yet difficult task, with there being no single accepted definition as to what constitutes a species, nor a set of criteria by which they should be delineated. Employing the General Lineage Concept somewhat circumvents these issues, as this concept allows multiple concordant lines of evidence to be used as support for species delimitation, where a species is defined as any independently evolving lineage. Genetically diverse groups have previously been identified within the only monotypic parastacid genus *Tenuibranchiurus*, but no further investigation of this diversity has previously been undertaken. Analysis of two mitochondrial DNA gene regions have previously identified two highly divergent groups, representing populations from Queensland (Qld) and New South Wales (NSW), respectively. Additional testing within this study of both mitochondrial and nuclear DNA through species delimitation analyses identified genetically diverse groups within these regions, which were further supported by lineage testing methods. The degree of genetic differentiation between Qld and NSW populations supports the recognition of two genera; with Qld retaining the original genus name *Tenuibranchiurus*, and NSW designated as *Gen. nov.* until a formal description is completed. Concordance between the species delimitation and lineage testing methods supports the presence of six species within *Tenuibranchiurus* and two within *Gen. nov.*. The recognition of additional species removes the anomaly of a single monotypic parastacid genus, and the methods used will contribute towards species identification within this taxonomically difficult group of organisms.

A novel genus and cryptic species harboured within the monotypic freshwater crayfish genus *Tenuibranchiurus* (Decapoda: Parastacidae).

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ABSTRACT

Identifying species groups is an important yet difficult task, with there being no single accepted definition as to what constitutes a species, nor a set of criteria by which they should be delineated. Employing the General Lineage Concept somewhat circumvents these issues, as this concept allows multiple concordant lines of evidence to be used as support for species delimitation, where a species is defined as any independently evolving lineage. Genetically diverse groups have previously been identified within the only monotypic parastacid genus *Tenuibranchiurus* but no further investigation of this diversity has previously been undertaken. Analysis of two mitochondrial DNA gene regions have previously identified two highly divergent groups, representing populations from Queensland (Qld) and New South Wales (NSW), respectively. Additional testing within this study of both mitochondrial and nuclear DNA through species delimitation analyses identified genetically diverse groups within these regions, which were further supported by lineage testing methods. The degree of genetic differentiation between Qld and NSW populations supports the recognition of two genera; with Qld retaining the original genus name *Tenuibranchiurus*, and NSW designated as *Gen. nov.* until a formal description is completed. Concordance between the species delimitation and lineage

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testing methods supports the presence of six species within *Tenuibranchiurus* and two within *Gen. nov.*. The recognition of additional species removes the anomaly of a single monotypic parastacid genus, and the methods used will contribute towards species identification within this taxonomically difficult group of organisms.

INTRODUCTION

Species are the fundamental unit of biodiversity; yet there has always been disagreement about criteria by which they should be recognised and the methods by which they should be delineated, with no general consensus reached thus far. The lack of one clearly accepted definition of a "species" creates obvious limitations, as what one person regards as a species may not be regarded as being so by another person, which is often further exacerbated by differences of opinion between fields of study. Employing the General Lineage Concept (GLC; de Queiroz 1998), where a species is defined as a metapopulation lineage evolving separately from other lineages, somewhat unites the various species concepts by allowing any evidence of lineage separation (and thus any property emphasised by the alternative concepts) to be used as evidence for species delimitation (de Queiroz 2007). Not only does this concept allow multiple lines of evidence to be used, but it also allows the evolutionary processes that have caused divergence between lineages to be examined.

Identifying species within freshwater crayfish has traditionally been undertaken through morphological examination. However, due to the tendency of crustaceans to contain both morphologically plastic or cryptic forms (e.g. Austin and Knott 1996; Murphy and Austin 2003; Silva *et al.* 2010; Breinholt *et al.* 2012), there has been an increasing shift towards the use of molecular methods to identify cryptic diversity (Hansen *et al.* 2001; Schultz *et al.* 2007; Mathews *et al.* 2008; Bentley *et al.* 2010; Dawkins *et al.* 2010; Sinclair *et al.* 2011). With the use of molecular techniques comes the potential for signatures of population-level and species-level histories to become confounded (Edwards 2008). This can occur when gene trees constructed from a single locus differ from the true genealogical history of a species (Sunnucks 2000; Hey and Machado 2003), although this problem can potentially be overcome by estimating gene trees from multiple unlinked loci. Using multiple loci from different areas of the genome (e.g. mtDNA and nuDNA) can account for the different patterns of evolution experienced by

each. For instance, mitochondrial alleles accumulate nucleotide substitutions several times faster than nuclear genes due to their lower N[e], thereby completing the coalescent process much faster and becoming diagnostic of taxa more rapidly (Sunnucks 2000).

Once a species tree has been inferred, additional testing is often undertaken to provide support for the proposed species' groups. A range of statistical analyses are available for testing species boundaries and, as there is currently no universally accepted way to define species, there are also a range of critiques on these methods (e.g. Sneath and Sokal 1973; Brower 1999; Wiens and Servedio 2000; Tautz *et al.* 2002; Wiens and Penkrot 2002; Lipscomb *et al.* 2003; Seberg *et al.* 2003; Sites and Marshall 2003; Tautz *et al.* 2003; Blaxter 2004; Ebach and Holdrege 2005; Will *et al.* 2005; Yang and Rannala 2010). Under the GLC, any evidence of lineage separation can be evidence for the existence of different species (de Queiroz 2007); as such, the identification of numerous corroborating lines of evidence (through the use of multiple tests) can be seen as lending support to any species boundaries that are defined. Therefore, although no single test is currently universally accepted, the apparent need to choose a particular method is circumvented by using a selection of techniques and multiple gene regions as, under the GLC, concordance between multiple lines of evidence is seen as increasing the rigour of species delimitation.

The parastacid crayfish genera are generally highly speciose, with novel species and genetically diverse groups commonly found (e.g. Coughran 2005; Hansen and Richardson 2006; Coughran *et al.* 2012; Furse *et al.* 2013). The most notable exception to this is the genus *Tenuibranchiurus*, which represents the smallest crayfish in the Parastacida[e]. Although it has previously been highlighted as containing genetically diverse groups (see Horwitz 1995; Dawkins *et al.* 2010), this genus as currently recognised contains only the single described species *Tenuibranchiurus glypticus* Riek (1951), and is the only monotypic parastacid genus. *Tenuibranchiurus* falls within a monophyletic clade containing the other Australian burrowing crayfish (*Gramastacus*, *Geocharax*, *Engaewa*, *Engaeus sensu stricto*, and *Engaeus lyelli* (distinct from other *Engaeus* species, *sensu* Schultz *et al.* 2009)) (Horwitz 1988), and is endemic to the central-eastern coast of Australia, spanning south-east Queensland (Qld) and north-eastern New South Wales (NSW) (Fig. 1). It was first suggested by Horwitz (1995), on the basis of electrophoretic and geographical differences, that previously unrecognised genetic diversity

existed within the genus. Subsequently, two genetically divergent groups were identified within this region by Dawkins *et al.* (2010), both of which showed considerable genetic variability within them. The two groups identified aligned with populations from Qld and NSW, respectively, and were suggested to represent species that diverged as a result of long-term historical geographic isolation (Dawkins *et al.* 2010). This study seeks to quantify the genetic diversity present within *Tenuibranchiurus*, utilising molecular data across several gene regions and employing multiple species delimitation methods in order to determine the most likely species groups.

METHODS

A total of 133 specimens were collected across 16 field localities, including the type locality for *T. glypticus*. All specimens from this study were collected under permits WITK08599510, WISP08599610, and TWB/01/2011 issue by the Department of Environment and Resource Management. DNA was extracted from specimens preserved in 70% ethanol using a variation of the cetyltrimethyl ammonium bromide/phenol-chloroform extraction protocol (Doyle and Doyle 1987). Two mitochondrial gene regions: cytochrome oxidase subunit 1 (COI; primers CRCOI-F/R (Cook *et al.* 2008)) and 16S rDNA (16S; primers 16S-ar/br (Palumbi *et al.* 1991)); and three nuclear gene regions: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primers G3PCq-157/981 (Schultz *et al.* 2009)), histone-3 (H3; primers H3-AF/AR (Colgan *et al.* 1998)), and arginine kinase (AK; primers AKcray-F/R (J.W. Breinholt, unpublished data)); were amplified. Sequences were edited using Sequencher 4.9 (GeneCodes 2009) and aligned using the MUSCLE addition in MEGA5 (Edgar 2004). Alignments were then checked and edited by hand if necessary.

Phylogenetic Analyses

A total of 127 *Tenuibranchiurus* samples were sequenced for the COI gene fragment, 59 for 16S, 93 for GAPDH, 57 for H3, and 46 for AK (Table 1). Additional specimens from the genera *Gramastacus*, *Geocharax*, *Engaeus*, *Engaewa*, and *Cherax* were also sequenced for inclusion as outgroups. Where sequences from these outgroups could not be obtained (i.e. due to non-amplification), alternative sequences were retrieved from GenBank (details in Table S1). Sequences obtained in this study were deposited in GenBank under accession numbers KX669691-KX670093, KX753349.

Table 1. Number of *Tenuibranchiurus* specimens analysed for each gene fragment from each of the sampled localities, as well as outgroup sequences included (see Table S1 for sequence details).

State	General Locality	Location ID	Number of specimens analysed				
			COI	16S	GAPDH	H3	AK
Qld	Kinkuna National Park	KNP	-	1	-	-	-
	Hervey Bay	HB	1	4	-	4	4
	Maryborough	MAR	10	5	9	5	3
	Tuan State Forest (North)	TSFN	2	2	-	-	-
	Tuan State Forest (South)	TSFS A	4	1	4	1	1
		C	14	3	12	4	4
		E	4	2	4	2	2
		F	3	1	3	1	1
		G	4	-	4	-	-
		H	1	-	1	-	-
	Tewantin	TEW	7	3	7	4	4
	Lake Weyba	LW	7	4	7	5	4
	Eumundi	Eu	-	1	-	-	-
	Mooloolaba	Moo	-	1	-	-	-
	Beerburrum	BER	7	2	5	2	2
	Type Locality	TL1	-	1	1	-	-
		TL2	-	2	1	1	1
		TL3	1	2	-	-	-
	Bribie Island	BRB1	-	-	1	-	-
		BRB2	4	-	-	-	-
		BRB	6	6	-	6	6
	Gold Coast	GC1	8	3	5	5	3
		GC2	7	3	6	4	3
NSW	Lennox Head	LH	13	4	10	4	3
	Broadwater National Park	BNP1	13	4	9	4	2
		BNP2	2	1	2	1	-
	Lake Hiawatha	LakeH	9	3	4	5	4
Total			127	59	95	58	47
	Gramastacus spp.		6	10	4	7	4
	Geocharax spp.		3	4	3	1	1
	Engaeus spp.		2	2	2	3	1
	Engaewa spp.		3	3	3	3	2
	Cherax spp.		1	1	1	1	-
Total including outgroups			142	79	108	73	55

Degree of molecular divergence

Preliminary analyses of both ~~the~~ individual and combined gene trees showed a prominent separation between Qld and NSW populations. In light of this, genetic distances between Qld and NSW populations, distances between these two groups and the outgroups, and distances between the outgroups were calculated using both COI and 16S data to compare the degree of separation. These distances were calculated in MEGA5 (Tamura *et al.* 2011) using the net between group mean distances with 1000 bootstrap replicates (gamma distribution with shape parameter = 1, Maximum Composite Likelihood (MCL) model; positions containing gaps and missing data were eliminated).

Species Delimitation

Two types of analyses were used to obtain a best-estimate of the species-level lineages present within *Tenuibranchiurus*; namely, groupings identified through use of a combined gene tree, and intra- versus inter-cluster variation through Φ_{ST} analysis. A combined gene tree analysis was chosen over individual gene trees because, although preliminary phylogenetic analyses performed on the individual gene regions suggested that there were multiple genetic groups within *T. glypticus*, statistical support was not always strong for all genes. Therefore, in order to increase the strength of the phylogenetic signal, and thus support for branching patterns, the five genes were combined and analysed as a single data set for phylogenetic reconstructions.

Combined gene tree

Combined gene trees were inferred using both Maximum Likelihood (ML) and Bayesian analyses. Specimens were included in the data set if they were sequenced for at least four of the five genes (see Table S1). The program RAxML v. 7.4.4 through the CIPRES Science Gateway (Miller *et al.* 2010) was used to infer the ML tree, and MrBayes v. 3.2.0 (Ronquist *et al.* 2012) for the Bayesian tree. Within the ML analysis, each gene was entered as a separate DNA-partition, the GTR+CAT model used, and bootstrapping automatically halted. For the Bayesian analysis, each gene was entered as a separate partition and the following parameters set; two replicate Markov chain Monte Carlo (MCMC) analyses with four chains in each analysis (one cold, three heated), the statefreq, revmat, shape, and pinvar all unlinked, the ratepr set as variable, and the analysis set to stop when the standard deviations of the partition frequencies (SD) <0.0099 (all effective sample size (ESS) values >100, PSRF+ ≈1.000, and the final Ngen

was 1,715,000). The same analysis was performed at least twice to verify topological convergence and homogeneity of posterior clade probabilities between runs. The first 25% of samples were discarded as burnin, with the resulting trees visualised using the program Figtree v. 1.4.0 (Rambaut 2012).

Intra- versus inter-cluster variation

An analysis of molecular variance (AMOVA) was used to calculate variation within and among clusters of sequences, as implemented in Arlequin v. 3.1 (Excoffier *et al.* 2005). To determine what the most likely lineages were, the clades identified by the combined gene tree analysis, as well as additional splits evident within the tree that were deemed to plausibly represent lineages, were also tested, as well as groups based on the geographic division of populations (i.e. collection locality). The AMOVA calculates three statistics; Φ_{ST}, Φ_{SC}, and Φ_{CT}, all of which are based on both the haplotype frequency and genetic divergence. Φ_{ST} measures variation among all populations, and Φ_{SC} measures among populations within groups, and Φ_{CT} estimates variation among groups. It has been suggested that an F_{CT} value >0.95 can represent evidence for accurate species groupings (i.e. >95% of the genetic variation can be attributed to differences among groups) (Monaghan *et al.* 2005). Using the Φ_{CT} estimate as a surrogate for F_{CT} (as this estimate includes genetic divergence as well as haplotype frequency), this can provide an approach to delineate taxa based on population genetic analyses by interpreting the AMOVA results used to calculate intra- versus inter-cluster variation in a way analogous to F-statistics (Wright 1978). The criterion to determine the appropriate number of lineages using this method is where an increase in the number of suggested lineages does not appreciably increase the Φ_{CT} estimate for those lineages.

Testing of Lineages

In order to test the lineages that were identified using the species delimitation methods for species-status, two approaches were used; barcoding gap identification (*sensu* Hebert *et al.* 2004), and the K/θ method (*sensu* Birky 2013). Only the mitochondrial data were used to test the species hypotheses, as the nuclear gene sample sizes were limited and individually were not very informative; for instance, most of the nuclear gene trees contained numerous polytomies and thus could not be used to identify genetically divergent groups.

192

193 Barcoding gap

194 The genetic distances between the hypothesised lineages and between specimens for both COI
195 and 16S were calculated and visualised to determine whether a barcoding gap existed. As the
196 intent of this test was to provide support for, or refutation of, the lineage hypotheses formed
197 through the species delimitation methods, lineages were pre-defined based on those results and
198 genetic distances categorised as representing either intra- or inter-lineage distances. For the
199 purposes of this study, a barcoding gap was defined as a clear separation (or 'gap') between the
200 highest intra-lineage and lowest inter-lineage genetic distances measured between the suggested
201 lineages. Although a standard threshold has been suggested by Hebert *et al.* (2004) for
202 recognising distinct species (10× average intraspecific difference), this approach was not
203 followed as it has been shown that there are vastly different rates of divergence for both different
204 taxa and different genetic markers (Avise 2009). Rather, a recognisable distinction between the
205 inter- and intra-lineage distances was considered potential evidence for distinct species.
206 Analyses were undertaken for Qld and NSW specimens separately.

207

208 Relative divergences between genetic groups were calculated in MEGA5. To determine inter-
209 lineage divergence, the number of base substitutions per site was estimated from the net average
210 between groups of sequences and the diversity between specimens was determined by
211 calculating the number of base substitutions per site between each sequence, both using a MCL
212 model with 1000 replicates. The rate variation among sites was modelled with a gamma
213 distribution with a shape parameter of 1, with positions containing gaps and missing data
214 eliminated. This was performed for both COI and 16S, with all unique haplotypes included.

215

216 K/θ Method

217 The species delimitation hypotheses were also tested using the K/θ method (Birky *et al.* 2005;
218 Birky and Barraclough 2009; Birky *et al.* 2010). Although this method was originally developed
219 for asexually-reproducing organisms and termed the 4X rule, it has been further developed and
220 shown to be effective for the mtDNA region for sexually-reproducing organisms (Birky 2013).
221 This method provides a simple way of defining species groups based on specimens/populations
222 that form clusters (i.e. clades) that are separated by genetic gaps too deep to be ascribed to

223 random genetic drift within a species and, therefore, must be due to diversifying selection or
224 long-term physical isolation (Apte *et al.* 2007).
225
226 Using the groups from the species delimitation hypotheses, sister clades were identified and
227 statistical support for these was tested. Sequence divergences were estimated within (d) and
228 between each sister clade using uncorrected p-distances calculated in MEGA5. Nucleotide
229 diversity (π) was then calculated using $\pi = dn/(n-1)$, where n is the number of samples per clade.
230 Theta (θ) was then estimated as $\theta = 2Ne\mu$ (where Ne is the effective populations size and μ is
231 mutation rate per base pair per generation) by calculating $\pi/(1-4\pi/3)$ within each clade. If $d = 0$
232 (as it did for one clade in this study), then π can alternatively be calculated as $2/Ln(n-1)$, where L
233 is the length of the sequence. K was then calculated for each sister-clade comparison (using
234 MEGA5) as the uncorrected net between group mean distance, with this divided by the highest θ
235 in the comparison (as this is the more conservative approach) to provide K/θ. Where sister
236 clades were poorly defined in the tree, K was estimated between all potential sister clades in the
237 polytomy, with the clade of the lowest K considered to be the sister clade. Finally, if the K/θ
238 value was greater than 4, then the sister clades were accepted as different lineages.
239

RESULTS

Degree of Molecular Divergence

The genetic distances calculated between the Qld and NSW groups using COI and 16S were 16.0% and 12.7%, respectively (Table 2). These distances were as large as, or in some cases larger than, the distances calculated between these two groups and other closely related genera. Furthermore, some distances between pairs of the other genera were smaller than those between the Qld and NSW groups for both COI and 16S (e.g. *Geocharax* versus *Engaeus* = 13.7% and 6.7%, *Gramastacus* versus *Engaeus* = 11.7% and 8.1%; Table 2).

Table 2. Estimates of net evolutionary divergence between groups of COI (below diagonal) and 16S (above diagonal) sequences with a MCL model.

	Qld	NSW	*Geocharax*	*Gramastacus*	*Engaeus*	*Engaewa*	*Cherax*
Qld	-	0.127	0.14	0.161	0.101	0.175	0.24
NSW	0.160	-	0.113	0.117	0.072	0.191	0.24
Geocharax	0.156	0.164	-	0.129	0.067	0.212	0.257
Gramastacus	0.185	0.206	0.203	-	0.081	0.244	0.242
Engaeus	0.109	0.086	0.137	0.117	-	0.138	0.189
Engaewa	0.164	0.154	0.160	0.169	0.103	-	0.347
Cherax	0.256	0.256	0.261	0.294	0.195	0.228	-

Species Delimitation

Groups that are identified as potentially representing distinct species will be referred to herein as Lineages, and will form the groups to be analysed through lineage testing methods.

Combined gene tree

Although not all groupings were statistically supported, both the ML and Bayesian combined gene trees suggested the presence of multiple groups within Qld. Six clades were evident within the Qld populations, with the monophyly of all but two highly supported (as these were represented by single specimens). The first clade included Maryborough and some Tuan State Forest specimens (Lineage 1; BS 90%, Pp 1), and the second contained the remaining Tuan State Forest specimens as well as Bribie Island, Type Locality, and some Beerburrum specimens (Lineage 2; BS 96%, Pp 1). The two groups for which monophyly could not be established were represented by the remaining Beerburrum specimens (Lineage 3) and Hervey Bay (Lineage 4). The final two clades consisted of Tewantin and Lake Weyba specimens (Lineage 5; BS 100%,

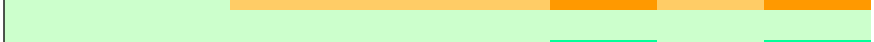
Pp 1) and Gold Coast specimens (Lineage 6; BS 100%, Pp 1). There was also some geographic structuring evident within each of the clades.

The two monophyletic clades evident within the NSW populations were strongly supported, and form Lineage 7 (Lennox Head) and Lineage 8 (Lake Hiawatha, Broadwater National Park 1 & 2) (Fig. 2). Although there was some structuring evident within Lineage 8, the branching patterns were very shallow and were therefore not explored as potential distinct lineages.

Intra- versus inter-cluster variation

A total of eight lineage arrangements was deemed plausible based on apparent genetic groupings and collection localities, and were tested using AMOVAs (Table 3). The process of assigning the potential lineages is outlined in Table S2, where a hierarchical approach was taken to split the tree into major genetic groups, minor genetic groups, and geographic localities. As there was no logical reason for combining the NSW lineages for the AMOVA analysis based on either the phylogenetic or geographic information, the NSW populations were considered to consist of the LH lineage and the LakeH/BNP lineage. Further testing, however, was considered appropriate to determine the lineages present within Qld. Figure 3 shows an increase in the Φ_{CT} estimate, with a plateau reached at six lineages for both the COI and 16S estimates. These six Qld lineages represent the most parsimonious arrangement of the specimens into lineages.

Table 3. Summary of possible lineages based on Φ-statistics for Qld specimens using COI and 16S data. See Table S2 for explanation of how potential lineages were determined. Where specimens from the same collection locality are split into two or more groups, details are included below the table for clarification.

Location ID	Number of potential lineages							
	2	3	5	6	8	10	11	13
MAR								
TSFN								
TSFSA[a]								
TSFSC[b]								
TSFSA								
TSFSC								
TSFSE								
TSFSF								
TSFSG								
TSFSH								
TL								
BRB								
BER[c]								
BER[d]								
HB								
LW								
TEW								
GC1								
GC2								
COI – Φ_{SC}	0.75848	0.73003	0.62052	0.40768	0.39226	0.49189	0.30985	0.16883
COI – Φ_{ST}	0.83245	0.82362	0.84592	0.82845	0.81145	0.80721	0.80969	0.80564
COI – Φ_{CT}	0.30627	0.34669	0.59395	0.71038	0.68975	0.62057	0.72424	0.83371
16S – Φ_{SC}	0.87218	0.84538	0.77989	0.53957	0.47467	0.56598	0.24716	0.43330
16S – Φ_{ST}	0.91463	0.91177	0.92051	0.91225	0.90574	0.90342	0.90123	0.89906
16S - Φ_{CT}	0.33209	0.42938	0.63887	0.80942	0.82056	0.77748	0.86880	0.92958

a = TSFSA;4
b = TSFSC;8,17,22
c = BER;1,2,5
d = BER;3,4,6,7

Species delimitation hypothesis

As the combined gene tree was inferred using only specimens that were successfully sequenced for at least four of the five genes, not all collection localities were represented on the tree (i.e. TSFN, KNP, Moo, Eu). Of these localities, only TSFN was represented in the AMOVA analysis, as the remaining localities were represented by a single sequence and therefore could not be included in the AMOVA. In order to assign these populations to a lineage for further testing, the individual gene trees and haplotype networks were examined and the localities were designated through the closest phylogenetic connection (data not shown). Both of the species delimitation methods suggested the presence of eight lineages (six in Qld and two in NSW; Table 4), and formed the lineages to be tested.

309 Table 4. Lineages assigned through two species delimitation methods and the final lineage hypothesis, for
310 Queensland and New South Wales localities. Dashes indicate where a population was not included.

Location ID	Combined gene tree	AMOVA	Lineage hypothesis
KNP	-	-	Lineage 1
TSFN	-		
MAR	Lineage 1	Lineage 1	
TSFS			
Moo	-	-	Lineage 2
TSFS			
TL	Lineage 2	Lineage 2	
BRB			
BER			
BER	Lineage 3	Lineage 3	Lineage 3
HB	Lineage 4	Lineage 4	Lineage 4
TEW	Lineage 5	Lineage 5	Lineage 5
LW			
Eu	-	-	
GC	Lineage 6	Lineage 6	Lineage 6
LH	Lineage 7	Lineage 7	Lineage 7
BNP	Lineage 8	Lineage 8	Lineage 8
LakeH			

311

312

313 **Testing of Lineages**

314 Barcoding gap

315 The COI data showed some overlap of the intra- and inter-lineage estimates within Qld, resulting
316 in no usable barcoding gap for lineage separation (Fig. 4A). Where the overlap occurred, the
317 low inter-lineage estimates were attributable to the Lineage 1 vs. Lineage 2 comparison, and the
318 high intra-lineage estimates were seen between specimens within Lineage 1. However, many
319 estimates between these two lineages fell in the higher range of the inter-lineage estimates as
320 well as the low range.

321

322 The 16S data for Qld populations showed a clearer relationship between lineages (Fig. 4C).
323 Although there was a very small overlap between the intra- and inter-lineage distances
324 (occurring between two specimens from Lineage 1), this represented an overlap of less than
325 0.01%. When the existence of this overlap was disregarded, there was a small gap at 2.8-3.0%.
326 However, despite there not being a distinguishable gap due to the overlap, identification of the
327 majority of lineages through the comparison of intra- and inter-lineage distances was clear and
328 distinguishable.

329

When the estimates within and between Lineage 1 and 2 specimens were removed from both the COI and 16S data (with the comparison between these two lineages and all other lineages remaining), a clear barcoding gap was seen in both data sets (Fig. 4B,D). For COI, the gap occurred between 1.7-4.7%, and between 0.9-3.5% for 16S. This shows that all other Qld groups (i.e. Lineage 3 through 6) represent clear lineages based on the barcoding approach using both COI and 16S data.

For NSW populations, there was a clear barcoding gap between the two lineages (i.e. Lineage 7 and 8), occurring between 1.5-6.6% for the COI data and 0.7-3.0% for the 16S data (Fig. 5).

K/θ method

The sister clades within Qld and NSW were tested using the K/θ method for a delimitation of eight lineages (six from Qld, two from NSW) using both COI and 16S data (Table 5). In some instances, sister clades that were defined by the lowest K-distance (as they were ambiguous based on the combined gene tree) differed between the COI and 16S datasets. In these cases, only the relevant K/θ comparison for the applicable gene was calculated.

Table 5. K/θ values for both COI and 16S for comparisons between sister clades within Queensland and New South Wales. Where specimens from the same collection locality are split into two or more lineages, details are included below the table for clarification. Dashes are used where sister clades differ between COI and 16S.

Sister Clade 1	Sister Clade 2	K/θ	
		COI	16S
Lineage 1	Lineage 2	0.78	1.41
Lineage 2	Lineage 1	0.78	1.41
Lineage 3	Lineage 1	-	1.67
	Lineage 5	6.99	-
Lineage 4	Lineage 5	7.18	-
	Lineage 6	-	32.84
Lineage 5	Lineage 6	6.71	-
	Lineage 2	-	4.92
Lineage 6	Lineage 5	6.71	8.24
Lineage 7	Lineage 8	16.03	6.48
Lineage 8	Lineage 7	16.03	6.48

Lineage 1 = MAR&TSFN&TSFSA (specimen 4) &TSFSC (specimens 8,17,22)
Lineage 2 = TSFSA-H (specimens 1-3,5-7,9-12,14,16,18-21,23-30) & BRB & TL & BER (specimens 1,2,5)
Lineage 3 = BER (specimens 3,4,6,7)

355 Lineage assignment

356 Although there was some ambiguity in the barcoding analysis of the Qld COI data regarding the
357 separation of Lineage 1 and 2, the 16S data showed support for the species delimitation lineage
358 hypothesis. Because of the deeper phylogenetic inferences provided by 16S in addition to the
359 fact that there were many genetic distances within and between Lineage 1 and 2 falling within
360 the expected distributions, the lineage hypothesis for Qld populations was considered supported
361 by this analysis (Table 6). The two NSW lineages were clearly separate based on both the COI
362 and 16S data and were therefore also supported (Table 6). In the K/θ analysis, all lineages were
363 supported by both genes with the exception of the split between Lineage 1 and 2 (both genes),
364 and Lineage 1 and 3 (16S) (Table 6).

365

366 Table 6. The species delimitation lineage hypothesis and two lineage testing methods, with the final
367 assignment of lineages for Queensland and New South Wales localities. Dashes indicate where a
368 population was not included.

Location ID	Lineage hypothesis	Barcoding gap	K/θ	Final lineage assignment
KNP TSFN MAR TSFS	Lineage 1	Lineage 1	- Lineage 1/2	Lineage 1
Moo TSFS TL BRB BER	Lineage 2	Lineage 2	- - Lineage 1/2	Lineage 2
BER	Lineage 3	Lineage 3	Lineage 3	Lineage 3
HB	Lineage 4	Lineage 4	Lineage 4	Lineage 4
TEW LW Eu	Lineage 5	Lineage 5	Lineage 5 -	Lineage 5
GC	Lineage 6	Lineage 6	Lineage 6	Lineage 6
LH	Lineage 7	Lineage 7	Lineage 7	Lineage 7
BNP LakeH	Lineage 8	Lineage 8	Lineage 8	Lineage 8

369

DISCUSSION

Phylogenetic Relationships

Based on a preliminary data set, Dawkins *et al.* (2010) highlighted the presence of two genetically divergent groups within *Tenuibranchiurus* and from this suggested the potential presence of two distinct species within the genus. The phylogenetic reconstruction of this study supports the presence of these two divergent groups, however, the larger data set used as well as the additional nuclear genes analysed suggests that the recognition of the two groups should be at a generic, rather than specific, level. Inclusion of the most closely related genera (i.e. *Gramastacus*, *Geocharax*, *Engaeus*, and *Engaewa*) in the analyses shows that the genetically divergent entities represented by the Qld and NSW groups each form monophyletic clades to the exclusion of all other genera. While the splitting of a monophyletic grouping into two genera is arguably arbitrary, the degree of divergence suggests it is warranted.

Although it is difficult to define what degree of separation is necessary between genera at a molecular level (Rach *et al.* 2008), based on the genetic distances presented there is strong support for a generic division. For instance, the genetic distance between Qld and NSW is larger than that seen between *Engaeus* and both *Geocharax* and *Gramastacus* for both gene fragments, and between *Engaewa* and both *Geocharax* and *Engaeus* for COI. Other genera also show smaller genetic distances when compared to either Qld or NSW than these two groups do with each other. Regardless of which genera were genetically closer to each other, the distance between Qld and NSW is at least as large as those between existing genera, thereby supporting their separation into two distinct genera.

Species Identification

Both of the species delimitation tests established the presence of the same eight lineages across Qld and NSW specimens of *Tenuibranchiurus*. Of these, Lineages 3 through 8 were highly supported by the two lineage testing methods used. However, support for the distinction between Lineages 1 and 2 was dependent upon the method and gene used. Using the barcoding approach, it has been found that recently diverged species are harder to distinguish than older species, with problems most likely attributable to incomplete lineage-sorting resulting in the lack of a barcoding gap (van Velzen *et al.* 2012 and references therein). Additionally, when using both the barcoding and K/θ methods, the high levels of genetic diversity found within each lineage (rather than low levels between them) may have resulted in these two lineages not being strongly supported. Alternatively, as has been found by other studies, retained ancestral variation between two recently-diverged clades may mask their current genetic isolation using the K/θ method, as divergence will follow a continuum and therefore no single percentage will work in every case (Druzhinina *et al.* 2012). Although this method has proven useful for other studies of sexually-reproducing organisms (e.g. Marrone *et al.* 2010; Leasi *et al.* 2013; Reniers *et al.* 2013), the results presented here suggest that it may not be suitable for delineating between some species where intraspecific diversity is high. In light of this, and considering the support shown by the species delimitation lineages suggested and the barcoding results, Lineage 1 and 2 are accepted as independently evolving lineages and, therefore, species.

As the currently described genus (i.e. *Tenuibranchiurus*) represents those specimens collected from within Queensland, specimens collected from New South Wales will belong to a newly proposed genus. Until a formal description is completed, the new genus will be referred to as *Gen. nov.*. The already described species *Tenuibranchiurus glypticus* (i.e. those populations grouped with the Type Locality) will retain this species name, with the remainder as follows:

Tenuibranchiurus sp. nov. 1: Maryborough, Tuan State Forest North and South, Kinkuna National Park

Tenuibranchiurus glypticus: Tuan State Forest South, Bribie Island, Type Locality, Beerburrum, Mooloolaba

Tenuibranchiurus sp. nov. 2: Beerburrum

Tenuibranchiurus sp. nov. 3: Hervey Bay

426 *Tenuibranchiurus* sp. nov. 4: Tewantin, Lake Weyba, Eumundi
427 *Tenuibranchiurus* sp. nov. 5: Gold Coast
428 *Gen.* nov. sp. nov. 1: Lennox Head
429 *Gen.* nov. sp. nov. 2: Lake Hiawatha, Broadwater National Park
430
431 **CONCLUSIONS**
432 Although genetic diversity within *Tenuibranchiurus* has previously been reported, no
433 quantification of this diversity has been undertaken. The multi-gene approach taken by this
434 study and use of several different analytical methods has identified not only several species
435 within the formerly monotypic *Tenuibranchiurus*, but ~~an addition~~ genus with two species ~~of its~~
436 ~~own~~. Although species identification of freshwater crayfish has traditionally been made through
437 morphological methods, ~~using~~ molecular methods ~~in this study allowed the potential pitfalls of~~
438 ~~plastic and/or cryptic morphological forms within crayfish to be avoided.~~ With the identification
439 of new species within the genus *Tenuibranchiurus* (and now also *Gen. nov.*), this has removed
440 the anomaly of a single monotypic genus within the parastacid crayfish, ~~and the methods used~~
441 ~~will contribute towards developing~~ a standardised method for dealing with species identification
442 within other freshwater crayfish.
443
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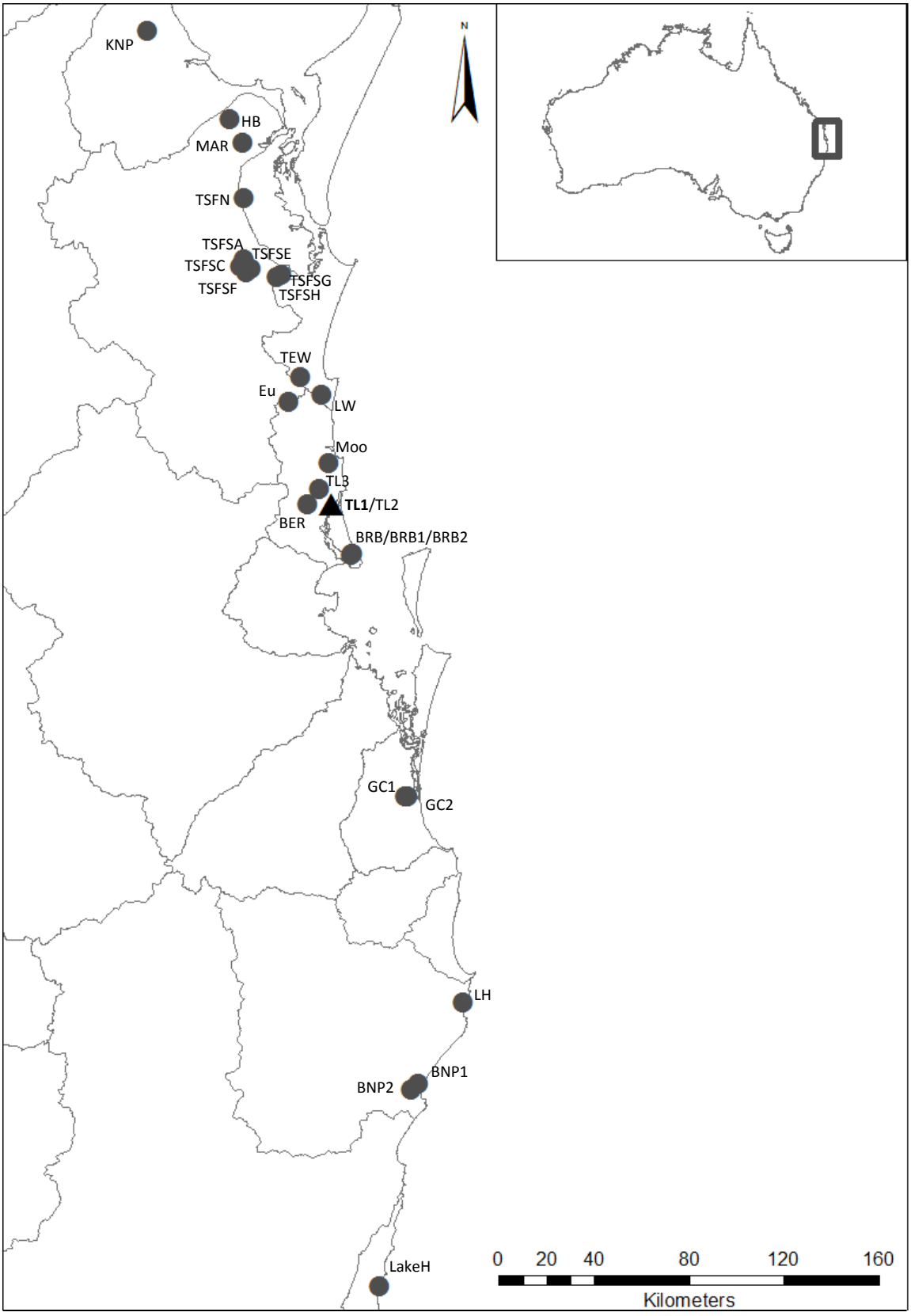

KNP
HB
MAR
TSFN
TSFSA
TSFSE
TSFSC
TSFSG
TSFSF
TSFSH
TEW
Eu
LW
Moo
TL3
TL1/TL2
BER
BRB/BRB1/BRB2
GC1
GC2
LH
BNP1
BNP2
LakeH
N
0 20 40 80 120 160
Kilometers

613 Figure 1. Localities where *Lamprologus nuibranchiurus* were collected during this study. The triangle and bolded
614 name denotes the Type Locality. Grey lines denote drainage boundaries. Refer to Table 1 for collection
615 details.

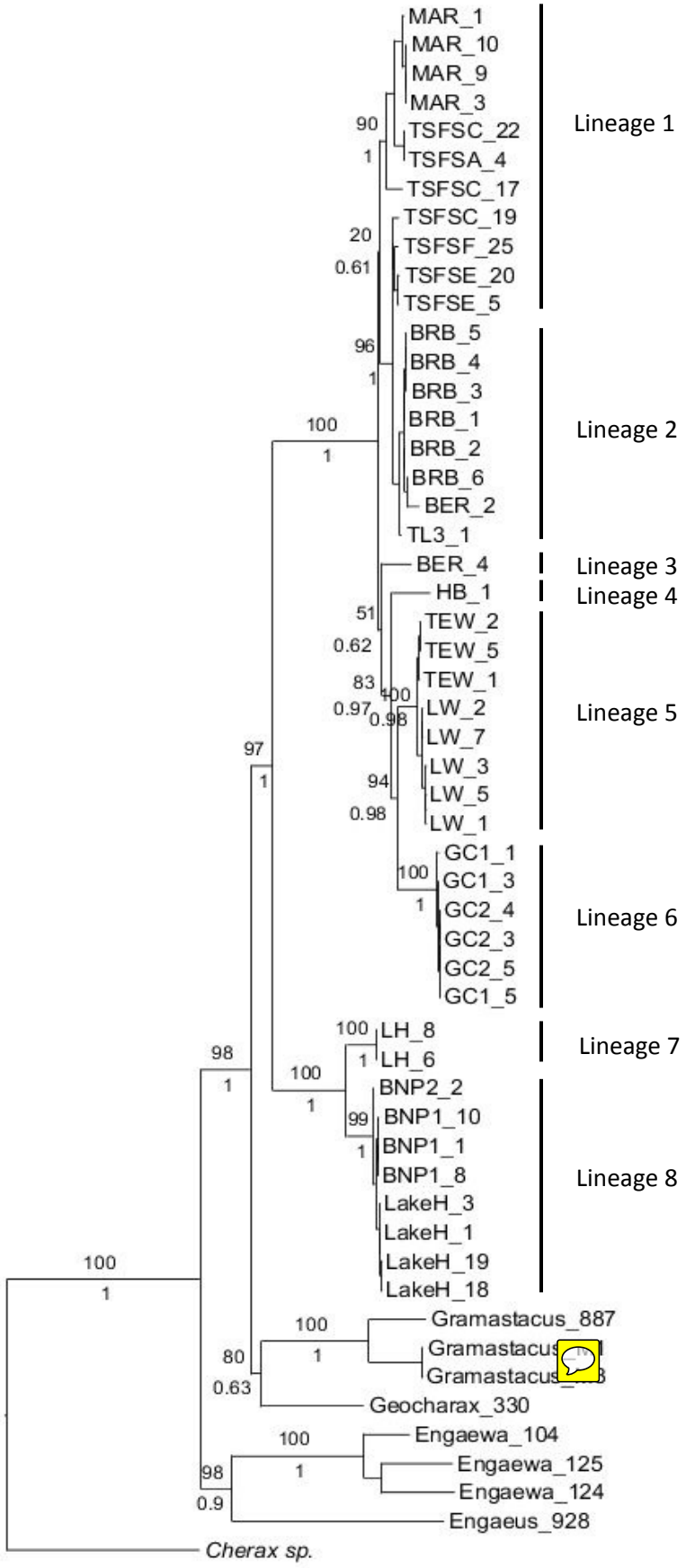

MAR_1
MAR_10
MAR_9
MAR_3
TSFSC_22
TSFSA_4
TSFSC_17
TSFSC_19
TSFSF_25
TSFSE_20
TSFSE_5
BRB_5
BRB_4
BRB_3
BRB_1
BRB_2
BRB_6
BER_2
TL3_1
BER_4
HB_1
TEW_2
TEW_5
TEW_1
LW_2
LW_7
LW_3
LW_5
LW_1
GC1_1
GC1_3
GC2_4
GC2_3
GC2_5
GC1_5
LH_8
LH_6
BNP2_2
BNP1_10
BNP1_1
BNP1_8
LakeH_3
LakeH_1
LakeH_19
LakeH_18
Gramastacus_887
Geocharax_330
Engaewa_104
Engaewa_125
Engaewa_124
Engaeus_928
Cherax sp.
Lineage 1
Lineage 2
Lineage 3
Lineage 4
Lineage 5
Lineage 6
Lineage 7
Lineage 8

Figure 2. Phylogram showing the proposed lineages for Queensland (Lineages 1 through 6) and New South Wales (Lineages 7 and 8). Bootstrap values are shown above the branches, and posterior probabilities below branches, for the major nodes.

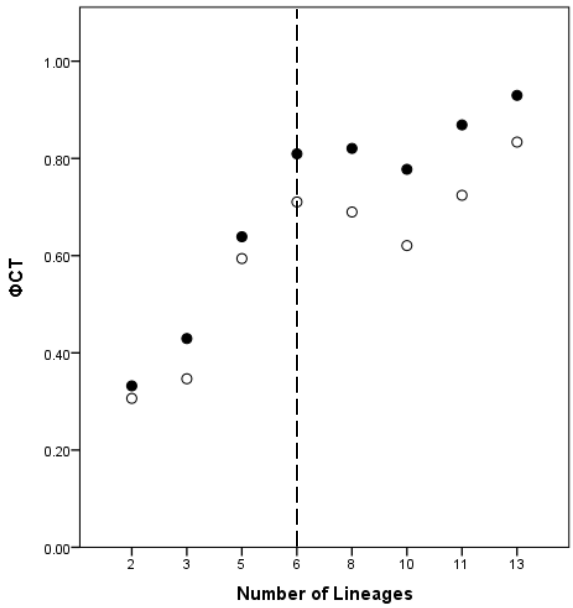


Figure 3. Φ_{CT} values for potential lineages for both COI (open circles) and 16S (black circles) for Queensland specimens. The dotted line indicates the most likely delimitation at six lineages.

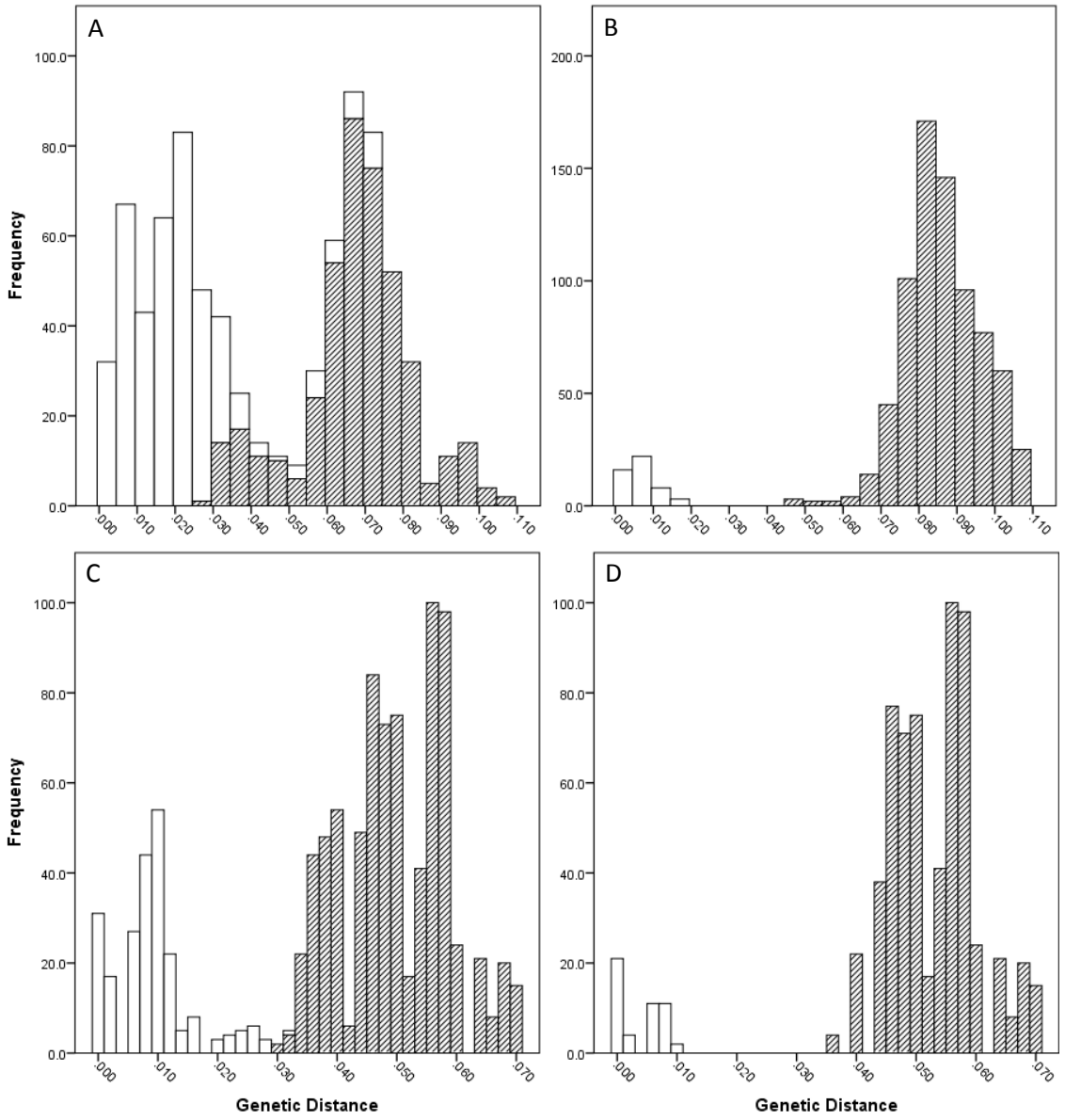
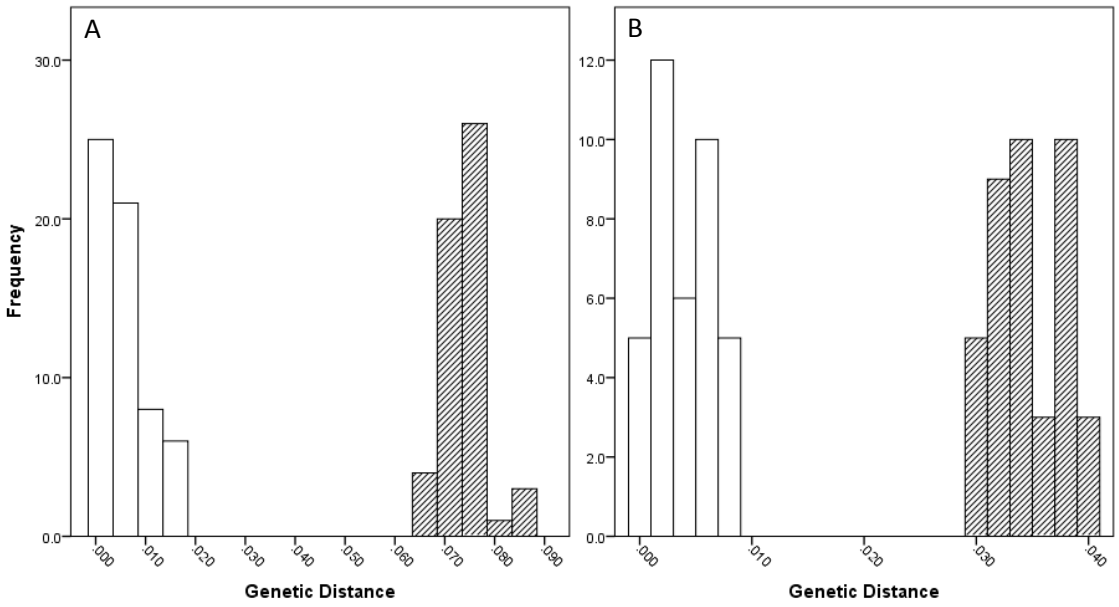


Figure 4. Intra- and inter-lineage genetic distance estimates (white and hashed, respectively) for Queensland lineages showing (A) COI estimates for all lineages, (B) COI estimates without comparisons between Lineage 1 and 2, (C) 16S estimates for all lineages, and (D) 16S estimates without comparisons between Lineage 1 and 2.



626 Figure 5. Intra- and inter-lineage genetic distance estimates (white and hashed, respectively) for New
627 South Wales lineages showing (A) COI and (B) 16S estimates for all lineages.