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A preliminary study of the utility of sequences from two mitochondrial protein coding genes for phylogeographic studies of marron (*Cherax cainii*) from southwest of western Australia.

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Abstract

Marron, *Cherax tenuimanus* and *Cherax cainii* (Austin) being two of the largest freshwater crayfish species in the world, are highly sought after by recreational anglers and form the basis of an expanding aquaculture industry in western Australia. This study investigates the potential for using mitochondrial protein coding gene regions for studying geographic patterns of genetic divergence among marron populations. Partial sequences of Cytochrome b (Cyt b) (471bp) and Cytochrome Oxidase I (COI) (600bp) gene regions were obtained from marron representing 13 populations from southwest Western Australia, and from Kangaroo Island, South Australia. Two data sets that derived from Cyt b and COI gene regions showed significant phylogenetic signal. Within *C. cainii*, twelve COI and ten Cyt b haplotypes were observed from the 14 locations with little intrapopulation diversity and with few haplotypes found at more than one location. Average divergence between species is Average divergence within *C. cainii* is 0.64% for Cyt b and 1.58% for COI gene region. Average divergence between *C. cainii* and *C. tenuimanus* is 12.17% and 5.89% for Cyt b and COI gene regions respectively. The phylogenetic analyses indicated that the two marron species, *C. tenuimanus* and *C. cainii* are quite divergent and provide evidence for geographic fragmentation in *C. cainii*. In *C. cainii*, the most significant phylogenetic structure was among samples from Western Australia's south coast river systems compared to the samples from the northern part of the species distribution which showed much less diversity. The study indicates that both the COI and Cyt b gene regions will be valuable for studying geographic population structure within the widespread species, *C. cainii*.

Introduction

The management of commercial, recreational and cultured fish and invertebrate species is benefiting from the greater availability of molecular genetic information for genetic studies (Ward and Grewe 1994). This information is essential for identifying evolutionary significant units, establishing management units (Moritz 1994a, b) and discovering cryptic species; all of which is vital for formulation of effective management

practices for sustainable exploitation and conservation of biodiversity. The ability to examine the genealogy or phylogeny of genes themselves rather than just estimate their frequencies has led to the development of the rapidly expanding field of phylogeography (Avise 2000). This approach allows genetic variation within and between populations to be interpreted in a geographical and historical context and allows distinguishing recent patterns of divergence due to recent isolation from historical events.

Species of marron, Cherax tenuimanus Smith (1912) and *C. cainii* Austin and Ryan (2002), are the largest freshwater crayfish in Western Australia (WA) and one of the largest freshwater crayfish in the world (Lawrence and Morrissy 2000). Marron are highly sought after by recreational anglers and are a popular candidate for aquaculture. As a consequence, marron have been extensively translocated (Morrissy 1978, Lawrence and Morrissy 2000). They have been introduced to several other states in Australia where feral populations have become established, as well as to a number of countries in both the Northern and Southern Hemispheres (Lawrence and Morrissy 2000). In Western Australia marron have thought to be restricted to a limited number of river systems in the extreme south-west of the state, but their distribution has now been substantially extended in recent times by translocations to both natural and man-made water bodies as a result of their popularity for aquaculture and recreational fishing (Lawrence and Morrissy 2000).

Molecular markers are generally the predominate method for assessing population genetic structure and measuring genetic diversity within and between populations of commercial fish and invertebrate species (Avise 1994, Johnson 2000). Most population genetic studies that utilise DNA sequencing survey the mitochondrial genome, given its rapid evolution, small effective population size and clonal inheritance (Avise 2000). This last aspect is especially important as it means that individual genotypes (haplotypes) retain genealogical information in the form of base substitutions.

Analysis of mitochondrial DNA sequences have been successfully used in number of freshwater crayfish studies to address a range of questions in the field of population genetics, taxonomy and phylogenetics (Crandall et al. 1995, 1999, Lawler and Crandall 1998, Ponniah and Hughes 1998, Hansen and Smolenski 2000, Munasinghe et al., 2003). A recent molecular systematic study by Munasinghe et al. (2003) of a range of Cherax species from the south west of Western Australia showed that for marron,

fragments amplified from the 12S and 16S showed little intraspecific variation while fragments amplified from the Cytochrome Oxidase I (COI) and Cytochrome b (Cyt b) gene regions were more variable. As Munasinghe et al. (2003) only examined five populations of marron over a limited geographic range, we extended this work by incorporating nine additional populations from throughout most of its geographic range in the south west of Western Australia using sequences from the COI and Cyt b gene regions. The objective of this study is to evaluate the effectiveness of sequences from these two gene regions to investigate genetic divergence among marron populations and between two marron species.

Materials and Methods

A. Collection of samples

Marron samples were obtained from 13 locations throughout the southwest of Western Australia. Samples were collected using baited traps. Either a leg was removed from marron in the field and placed in 90-95% EtOH or the whole crayfish were frozen in liquid N2 or dry ice and subsequently stored at –20°C in the laboratory. Sequences from a previous study (Munasinghe et al. 2003) for five sample sites (MAR, BEB, DON, WAR, INR) were previously submitted to GenBank under accession numbers AF 493626 – AF493631 and AF 492794 – AF 492799 for COI and Cyt b respectively. A sample of marron from a feral population from Kangaroo island was also included and a sample of C. quinquecarinatus Gray (1845) from the Canning River, Western Australia was used as an outgroup. The sample collection sites are given in the Figure 1.

B. DNA extraction and amplification of Mitochondrial DNA

Total DNA was extracted using the method outlined by Crandall et al. (1995) from muscle tissue from either marron legs or from the abdomen. An approximate 600 base pairs (bp) fragment of mitochondrial COI gene was amplified with the oligonucleotide primers CAF 5' CTACAAATCATAAAGATATTG 3' and CABR 5' CTTCAGGGTGACCAAAAAATC 3' (modified from Folmer et al. 1994). In addition an approximately 470 bp fragment of the mitochondrial Cyt b gene was amplified for these samples using YTF 5'TTACCTT GAGGACAAATATCAT 3' and YTBR 5' CACCTCCTAATTTATTAGGAA 3' primers (Munasinghe et al. 2003). PCR and sequencing reaction volumes and thermal-cycler parameters were as employed by Munasinghe et al. (2003).

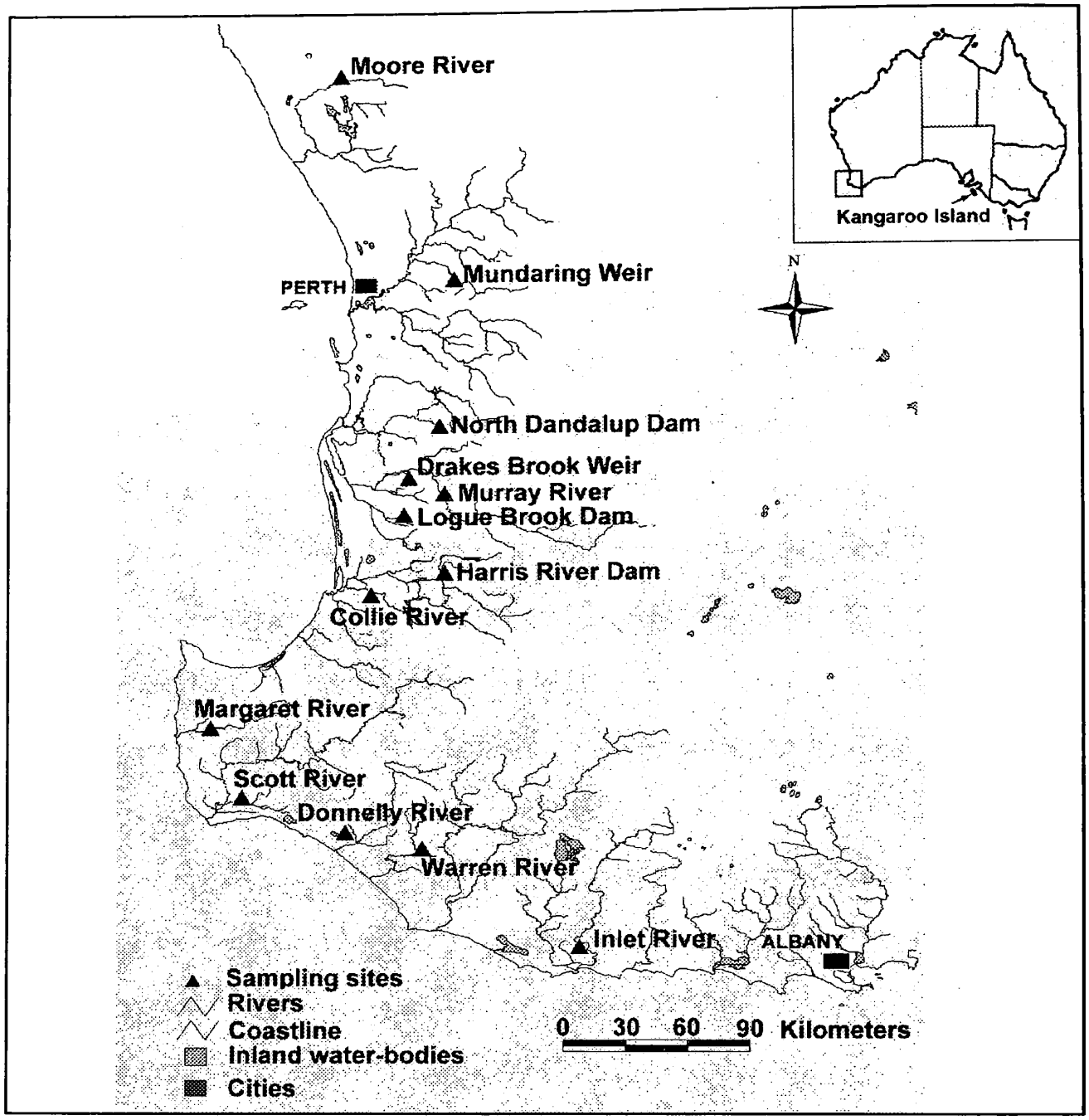

Figure 1. Sampling sites.

C. Data analysis and phylogenetic reconstruction

The sequence chromatograms were viewed using Edit View software and edited using the Seq-Pup program (Gilbert 1997). Sequences were aligned manually because no insertions or deletions were detected. Phylogenetic analyses were carried out using PAUP 4.0b10 (Swofford 2000) unless otherwise stated. Modeltest 3.04 (Posada and Crandall 1998) was used to obtain the most appropriate model of evolution for the two gene regions. Pair wise sequence divergence was calculated using the resultant model of nucleotide sequence evolution. Phylogenetic signal within data sets were assessed using the g1 (Hillis and Huelsenbeck 1992) statistics from the random tree distribution option of PAUP 4.0b10.

Phylogenetic analyses were conducted using the three different optimality criterion; Minimum Evolution (ME), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. The models of sequence evolution determined from Modeltest were employed during the ME and ML analyses. For MP and ML analyses a heuristic search option employing 10 random stepwise sequence additions and Tree Bisection and Reconnection (TBR) branch swapping. Confidence in the resulting relationships was assessed using the bootstrap procedure with 200 replicate data sets for ML and 1000 for ME and MP analyses. Amino acid sequences were obtained using the MacClade program (Maddison and Maddison 1992). Finally, Haplotype diversity (h) was calculated according to Nei (1987).

Results

Approximately 600 bp of the COI gene region and 470 bp of Cyt b gene regions were sequenced from 34 individual marron samples representing two species, *C. cainii* and C. tenuimanus, from 13 locations in the south west of Western Australia and one sample from Kangaroo Island. All new unique haplotype sequences for each gene region have been submitted to GenBank (Accession Numbers for COI AF 510181 – AF510187, for Cyt b AF 510176 – AF 510180). Sequence alignments for Cyt b and COI gene regions are given in Annex I and II.

The characteristics of the two gene regions are summarised in the Table 1.The proportion of variable sites was 17.83% for Cyt b gene region and 17.00% for COI gene region. Both data sets indicate significant phylogenetic signal for all taxa (g1 = -0.81 for Cyt b and g1 = -0.56 for COI, $P > 0.01$) as well as ingroup taxa (-0.79 for Cyt b and -0.55 for COI, $P > 0.01$). Low Tr/Tv ratios were observed for both gene regions at the intraspecific level (1.32 for Cyt b and 1.92 for COI) while substantially higher ratios at the interspecific level (5.19 for Cyt b and 2.51 for COI). Major differences can be seen in nucleotide divergence levels. The intraspecific divergence levels (uncorrected) ranged 0 – 1.49% for Cyt b gene region and from 0 – 3.67% for COI gene region. The interspecific divergence levels (uncorrected) ranged from 11.67 –12.33% for Cyt b region and 4.83 – 7.00% for COI gene region.

A total of 200 and 158 amino acid residues were obtained for the translated COI and Cyt b gene fragments respectively. These amino acid sequences appeared to be part of functional proteins as they lacked stop codons, frame shifts and deletions. Among the *C.*

cainii samples, 12 COI and 10 Cyt b haplotypes were observed with all individuals sampled from the same location having the haplotype with the exception of Logue Brook Dam (LOG1 and LOG2) (Annex I and II). In general the greatest haplotype diversity was observed among samples from along the south coast (h = 0.83 for Cyt b and COI) and the least among samples from the northern part (h = 0.64 for Cyt b and 0.81 for COI) of the species range.

Modeltest identified TrN + I and TrN + I + G as the most appropriate evolutionary models for Cyt b and COI gene regions respectively. For each data set, the three tree building methods produce identical tree topologies. The phylogenetic trees derived using the ME method with bootstrap values are given in Figures 2 and 3. Topologies derived from each data set clearly highlight the distinctiveness of the *C. tenuimanus* population from the *C. cainii* populations as the primary split in the phylogenetic tree. The trees indicated that the deepest phylogenetic divergence is among marron from the south coast rivers and the northern populations are more genetically homogenous.

Table 1. Characteristics of two gene regions

Characters	Cyt b	COI
Number of base pairs	471	600
% Variable sites		
All samples	17.83	17.00
Marron samples	13.16	8.83
A + T %	62.70	61.00
Transition/Transversion		
Intraspecific level	1.32	1.92
Interspecific level	5.19	2.51
% Average Divergence		
Intraspecific level	0.64	1.58
Interspecific level	12.17	5.89
Skewness		
All	-0.81	-0.56
Marron samples	-0.79	-0.55
Parsimony informative sites	21	34
Modeltest		
Optimum model of evolution	TrN + I	TrN + I + G
Gamma Shape parameter	Equal sites	0.65

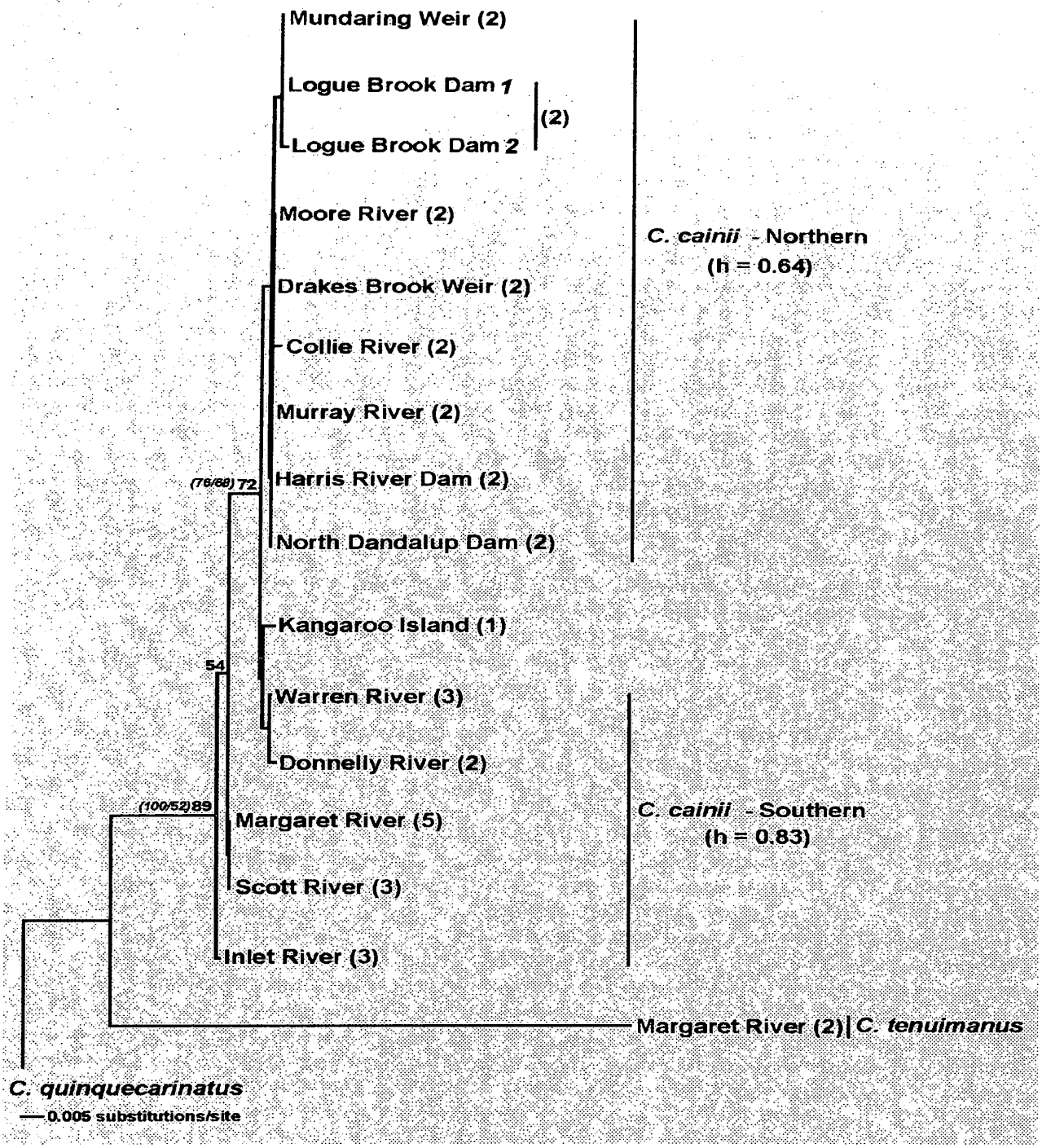

Figure 2. The Minimum Evolution tree derived from Cyt *b* data set estimated using TrN + I model of evolution. The bootstrap values (> 50%) are in bold are for ME analyses. Bootstrap values for MP and ML analyses are given in parentheses (MP/ML). Sample size is given in parentheses (next to sample locations) and italic numbers indicate the haplotype number. Haplotype diversity for northern and southern *C. cainii* populations is given by 'h'.

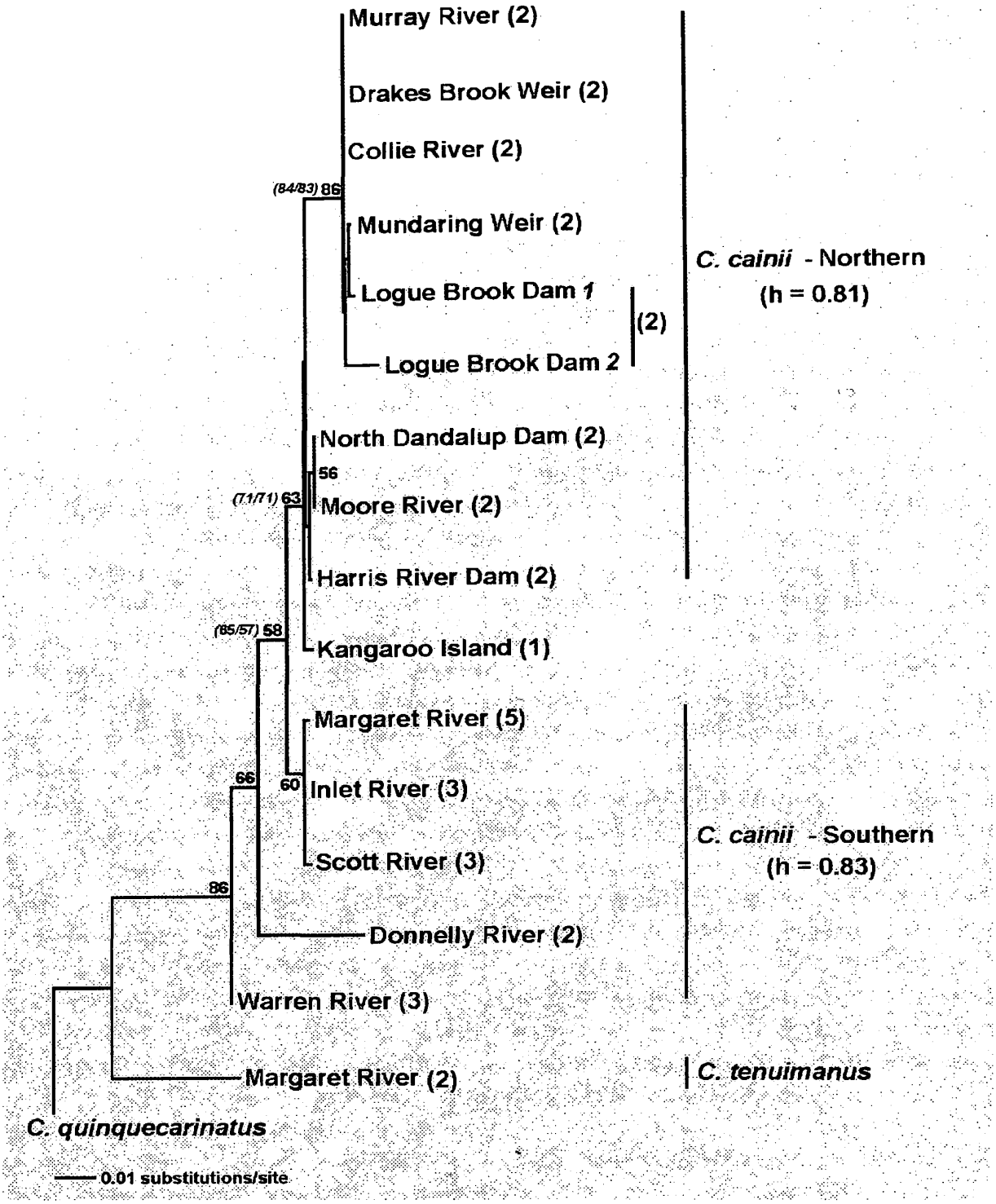

Fig. 3. The Minimum Evolution tree derived from COI data set estimated using TrN + I + G model of evolution. The bootstrap values (> 50%) are in bold are for ME analyses. Bootstrap values for MP and ML analyses are given in parentheses (MP/ML). Sample size is given in parentheses (next to sample locations) and italic numbers indicate the haplotype number. Haplotype diversity for northern and southern *C. cainii* populations is given by 'h'.

Discussion

This study demonstrates the potential usefulness of the mtDNA COI and Cyt *b* gene regions for investigating intraspecific relationships in freshwater crayfish. It is the first study to show geographic-based genetic diversity among populations of *C. cainii* sampled from throughout most of the species geographic range.

After the 16S gene, COI sequences are the most commonly employed mtDNA gene region for systematic studies of decapod crustaceans (Tam *et al.* 1996, Saver *et al.* 1998, Gopurenko *et al.* 1999). Very recently this gene region has been applied to studies of systematic relationships among species of freshwater crayfish (Hansen and Smolenski 2000, Taylor and Hardman 2002, Munasinghe *et al.* 2003). Although Cyt *b* gene sequences are widely used in systematic and population level studies in vertebrates (Hillis *et al.* 1996) this is one of very few studies to have used this gene region for studies of crustaceans. This may due in part to the lack of available primers and because of amplification of nuclear translocated copies of the gene (pseudogenes) (Zhang and Hewitt 1996).

While the possibility of pseudogenes amplification in this study cannot be completely discounted, all the fragments amplified (for both COI and Cyt *b*) appeared to be part of functional proteins as they code for amino acid sequences without stop codons, indels or frameshift. Further, the phylogenetic relationships among samples established from the two gene regions did not generate any unexpected relationships. Two gene regions indicate that the COI and Cyt *b* genes can be used to investigate the population and conservation genetics and phylogeography of *C. cainii*. The need for such studies are assuming greater importance with the increasing realisation of the vulnerability of freshwater crayfish to a range of threatening processes including intra-specific translocations (Austin and Ryan 2002). *C. cainii* is especially vulnerable to translocation as it is subject to a popular recreational fishery and is frequently translocated for aquaculture purposes (Morrissy 1978).

The finding that *C. cainii* is genetically heterogenous for COI and Cyt *b* is highly significant as previous studies using allozymes or other mtDNA sequences from the 16S and 12S rRNA gene regions showed no or minimal genetic variation (Austin and Knott 1996, Austin and Ryan 2002, Nguyen *et al.* 2002, Munasinghe *et al.* 2003). Although the sample sizes are low in this study there are some general observations that can be made. First it is noteworthy that almost all diversity is between rather than within populations of *C. cainii*. Considering both gene sequences, only one population (Logue brook Dam) of 13 sampled for which 2 or more individuals were sequenced was polymorphic. Out of

the 14 populations sampled, 8 (Cyt *b*) or 9 (COI) had haplotypes unique to that site. This result is similar to allozyme studies of other species of freshwater crayfish, which show low levels of intra-population variation and high levels of inter-population variation (Campbell *et al.* 1994, Austin 1996, Austin and Knott 1996, Avery and Austin 1997).

A second potentially significant observation is that the haplotype diversity of marron in the northern part of its range is less than that observed among marron from south coast rivers. This may reflect the extension of the distribution of the marron northward by translocation as suggested by Morrissy (1978). However, other explanations are possible such as natural range expansion from the southwest, ongoing or recent gene flow or persistence of ancient haplotypes in south coast populations. Further studies are required with greater sampling intensities and utilising appropriate statistical techniques such as nested clade analysis (Templeton 1998) to discriminate among these various possibilities.

Thirdly, the finding of inter-population genetic diversity within *C. cainii* has implications for genetic improvement studies and the conservations of genetic resources within this species. Population genetic information can be used to establish 'genetic baseline' information to help maintain genetic diversity and the potential for obtaining a positive response to selection (Lawrence and Morrissy 2000). However the potential for the escape into the wild of any genetically improved marron would need to be evaluated with great care given the vulnerability of wild crayfish to the introduction of non-indigenous forms (Austin and Ryan 2002). Thus, an important extension of this project is to quantify more fully the genetic variation within populations of *C. cainii* in order to identify populations in need of conservation on the basis of their isolation and genetic distinctiveness.

Thus in conclusion, given the increasing interest in commercial exploitation of *C. cainii* and the awareness of conservation issues affecting freshwater crayfish there is an urgent need for further research to determine the natural distribution of marron (Morrissy 1978) and the occurrence and distribution of unique genetic forms through more comprehensive sampling and genetic analysis. In this regard it is apparent that sequencing of the mitochondrial gene regions used in this study or other more variable regions (e.g. control region) coupled with phylogeographic analyses will be very useful. These kinds of data and analyses will be essential for effective decision making in relation to the management and conservation of genetic resources within natural stocks of marron and the sustainable exploitation of *C. cainii* in the southwest of Western Australia.

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