



The evolutionary diversification of the Centropagidae (Crustacea, Calanoida): A history of habitat shifts

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ABSTRACT

The copepod family Centropagidae is widely distributed and occurs in marine, estuarine, freshwater, and inland saline settings. Molecular phylogenies based upon the 16S and 28S genes demonstrate a complex biogeographic history, involving at least five independent invasions of continental waters from the sea. The first colonization was ancient, likely into part of Gondwanaland, and resulted in an inland radiation in southern genera via both vicariance and subsequent habitat shifting among different types of continental waters. Species occupying saline lakes are nested within freshwater clades, indicating invasion of these habitats via fresh waters rather than directly from the ocean or from epicontinental seas. In contrast with the great southern clade, all of the remaining continental invasions are northern, species poor, and quite recent, perhaps even Pleistocene. Long-lived evolutionary euryhalinity, a high propensity for inland invasion, continental vicariance, and *in situ* radiation within single continents have all played major roles in the diversification of the centropagids.

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1. Introduction

A key issue for understanding the origins of freshwater biodiversity is to identify the relative roles of repeated invasion from marine environments vs. diversification within continental waters. Indeed, variation in species richness among freshwater copepod groups is associated with the number and timing of inland invasions (Boxshall and Jaume, 2000). However, direct comparisons of taxonomic diversity can be problematic when studying diversification, because taxa of the same rank may be of different evolutionary ages or may not be monophyletic. Thus, investigations of phylogenetic relationships in clades having both marine and freshwater members are needed to provide insight into the origins of continental lineages, and such an approach has been used to un-

vel the histories of several enigmatic freshwater groups (e.g. Väinölä et al., 2001; Audzijonytė et al., 2005; Dooh et al., 2006).

The calanoid copepod family Centropagidae, a relatively large group consisting of ~110 species in 13 genera (Bayly, 1992a; Boxshall and Halsey, 2004), inhabits a great variety of aquatic environments and has long been of biogeographic interest (e.g. Bayly, 1964). The genus *Centropages* contains 29 oceanic species, while the other 12 genera occupy coastal marine, estuarine, freshwater, and inland saline habitats, or a combination of these (see Fig. 1). Understanding the role of habitat shifts in the diversification of this family involves two main questions: identifying the number of invasions of continental waters and understanding the frequency and order in which habitat shifts typically occur. Four studies in particular have investigated these questions in depth, presenting specific predictions about phylogenetic relationships in the centropagids that will be examined in this study.

Boxshall and Jaume (2000) suggested that there have been at least five separate invasions of freshwaters within this family,

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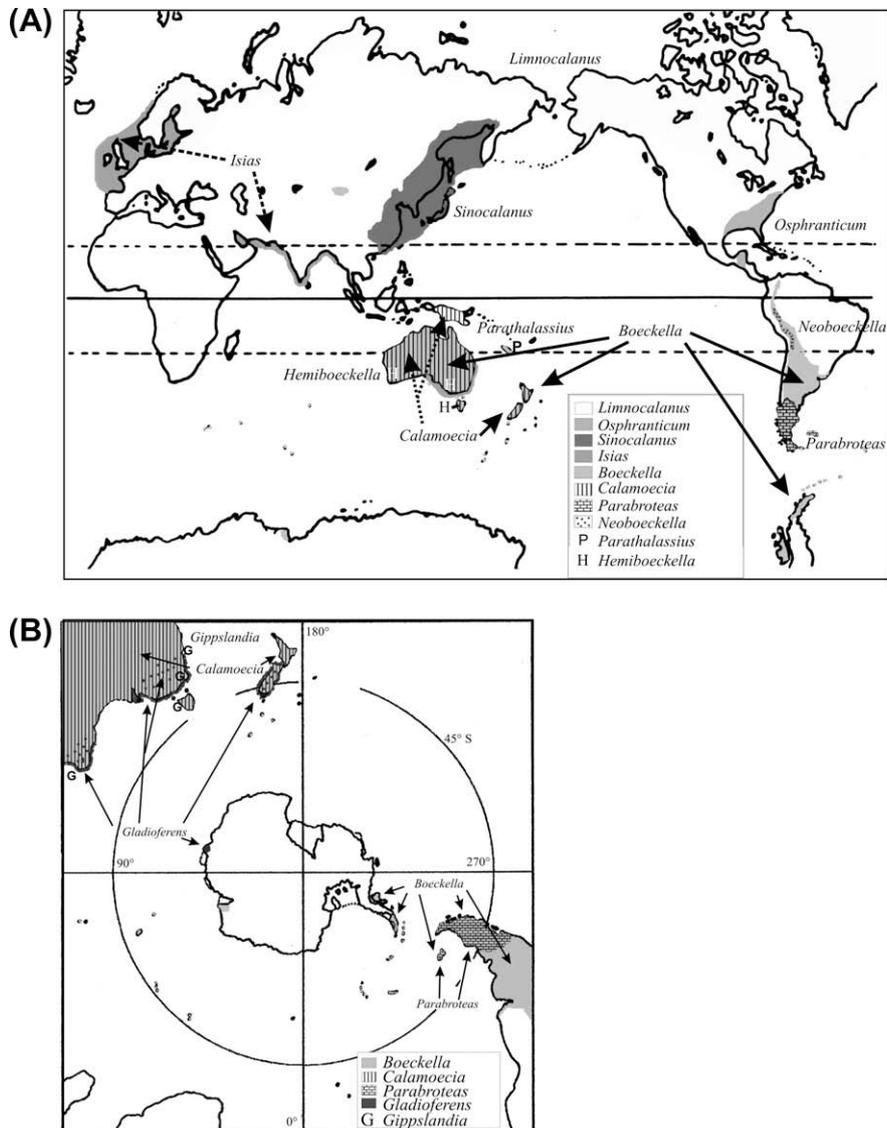


Fig. 1. Distribution of genera of the calanoid copepod family Centropagidae. (A) Global view of most generic distributions. *Centropages* is widely distributed in the world's oceans. (B) South polar view, detailing circum-Antarctic distributions. Details of habitat occupancy are as follows: *Boeckella* – inland waters, including lakes, ponds, rivers, and saline lakes in Australia, New Zealand, South America, and Antarctica; *Calamoecia* – inland fresh and saline water bodies in Australia, New Zealand, and New Guinea; *Centropages* (not shown) – exclusively marine, widely distributed in coastal waters; *Gippislandia* – estuaries of Australia; *Gladioferens* – freshwaters and estuaries in Australia, New Zealand, and Antarctica; *Isias* – estuarine and coastal marine waters off Europe, South Asia, and Australia; *Hemiboeckella* – freshwaters in parts of Australia; *Limnocalanus* – fresh, estuarine, and marine waters of the Holarctic; *Neoboeckella* – freshwaters in the Altiplano Andean region; *Osphranticum* – freshwaters of southeastern North America and Central America; *Parabroteas* – freshwaters in southern South America; *Parathalassius* – freshwaters of New Caledonia; *Sinocalanus* – freshwaters and coastal marine areas of East Asia. The arrows simply designate associations between the distributions and genus names.

two of which they count as “colonizations” (due to subsequent inland diversification) and three of which are categorized as “incurSIONS” (invasion without speciation). The origin of the 60 or so continental species of the southern hemisphere, belonging to the genera *Boeckella*, *Calamoecia*, *Hemiboeckella*, *Neoboeckella*, *Parabroteas*, and *Parathalassius*, was attributed to a single, ancient colonization event of Gondwanaland. *Sinocalanus* was proposed to have colonized Asian estuarine and freshwaters more recently, likely in the Pleistocene, where it subsequently diversified into five species. By contrast, *Osphranticum* is a monotypic North American freshwater genus, and *Limnocalanus johanseni* and *L. macrurus* are each distributed in both coastal marine areas and freshwaters in the Holarctic, representing three recent, independent incurSIONS without subsequent diversification. However, a lack of phylogenetic knowledge for the centropagids has precluded tests of these scenarios. In particular, the monophyly of the putative Gondwanan clade has not been established.

Bayly (1964) and Bayly and Boxshall (2009) propose a consistent directionality of habitat shifts in the centropagids, with transitions running from marine to estuarine to freshwater to inland saline environments. If correct, and if major shifts are relatively rare, the habitat distributions on a phylogeny should reflect these transitions. For example, the inland saline species (which are *Boeckella poopoensis*, *Calamoecia salina*, *C. clitellata*, and *C. trilobata* (Bayly and Boxshall, 2009)) are expected to be phylogenetically nested among freshwater species. By contrast, Maly (1996) argued that *Calamoecia* lineages now found in the inland saline waters of Australia trace their history of habitat occupancy directly to the sea, without having passed through freshwaters. Maly (1996) proposed that two important traits shared between *C. salina* and marine centropagids (osmotic conformance and the shedding of eggs directly into the water) are plesiomorphic rather than convergent or atavistic. Moreover, the geological history of Australia has included marine incurSIONS, epicontinental seas, and a long presence

of saline lakes (Bayly, 1967). Under Maly's (1996) scenario, freshwater and inland saline members of *Calamoecia* are expected to demonstrate both reciprocal monophyly and a long history of evolutionary isolation.

Here, expanding upon our previous extended abstract (Adamowicz et al., 2007), we use a molecular phylogenetic approach to evaluate these hypotheses and to characterize the relative roles of invasion from the sea, continental vicariance, and shifts among different habitats in the diversification of the centropagids.

2. Materials and methods

2.1. Sampling

Copepod samples were collected using hand nets or plankton tows in a large variety of localities, including sites in Australia, New Zealand, Argentina, Bolivia, Canada, the United States, and Japan (see Table 1). All material was preserved in 95% ethanol. In total, 30 species were available for analysis, including representatives of 8 of the 13 recognized genera: *Boeckella* (16 of 41 known species, including the sole saline lake representative), *Calamoecia* (6 of 15 species, including one saline lake species), *Centropages* (2 of 29), *Gladioferens* (1 of 6), *Limnocalanus* (2 of 2), *Osphranticum* (1 of 1), *Parabroteas* (1 of 1), and *Sinocalanus* (1 of 5) (diversity figures from Bayly (1992a,b) and Boxshall and Halsey (2004)). Five centropagid genera were unavailable for analysis: *Gippislandia* (which contains a single known species), *Hemiboeckella* (three species), *Isias* (three species), *Neoboeckella* (two species), and *Parathalassius* (one species). *Gippislandia* is a monotypic genus from Australian estuaries, while all three *Hemiboeckella* species are found in temporary environments in Australia and Tasmania. The members of *Isias* are generally rare and appear in coastal marine and brackish waters. *Neoboeckella* species are restricted to ephemeral freshwaters in the South American Altiplano. And finally, the monotypic genus *Parathalassius* from New Caledonia has not been collected again since its discovery. Specimens of *Karukinka fueguina* Menu Marque, 2002 were also unavailable, but this species had a COI (cytochrome *c* oxidase subunit I) sequence very similar to *Boeckella poppei* and may represent an atavistic form of that species or hybrid (see Adamowicz et al., 2007) or intersex individuals (Bayly and Shiel, 2008), possibilities which require further investigation. Distributions of all other centropagid genera are shown in Fig. 1. Three individuals of *Pseudodiaptomus inopinus* (part of the calanoid family Pseudodiaptomidae) were designated as the outgroup.

2.2. DNA extraction and sequencing

Phylogenetic analysis was based upon two ribosomal genes, one mitochondrial (16S) and one nuclear (28S). Fragments of 16S were PCR-amplified using universal primer pair 16Sar and 16Sbr (Palumbi, 1996), while 28S fragments were amplified using primers 28S-F and 28S-R (Schnabel and Hebert, 2003).

Whenever possible, we sequenced at least two individuals per species per locality for 16S and at least one individual per species for the slower-evolving 28S gene. DNA was extracted from individual whole animals into a 50 μ L volume per animal using either the proteinase-K protocol of Schwenk et al. (1998) or the NucleoSpin[®] 96 tissue kit (BD Biosciences Clontech), which was used according to manufacturer instructions except with overnight incubation and final elution in H₂O. PCR reactions were performed according to standard protocols (based upon Palumbi, 1996), with each 50- μ L reaction consisting of 3–5 μ L of the extracted DNA homogenate, 5 μ L of 10 \times PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl), 0.2 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and

1 unit of *Taq* DNA polymerase. A “touch-down” PCR thermal regime (Palumbi, 1996) was used, consisting of: one cycle of 1 min at 94 $^{\circ}$ C; 10 cycles of 1 min at 94 $^{\circ}$ C, 1.5 min at 60/58/56/54/52 $^{\circ}$ C (two cycles each), and 1.5 min at 72 $^{\circ}$ C; 25 cycles of 1 min at 94 $^{\circ}$ C, 1.5 min at 50 $^{\circ}$ C, and 1.5 min at 72 $^{\circ}$ C; and finally 5 min at 72 $^{\circ}$ C. PCR products were electrophoresed in 2% agarose gels infused with ethidium bromide and visualized with UV light. The desired fragment was excised, purified using the Qiaex II (Qiagen) kit, and subjected to dye terminator sequencing (30 cycles, 55 $^{\circ}$ C annealing) using the Big Dye Terminator (version 3) sequencing kit (ABI Prism). Products obtained during early phases of the project were sequenced in one direction using primers LCOI490 and 28S-F on an ABI 377 automated sequencer (Applied Biosystems), but most samples were sequenced in both directions.

2.3. Phylogenetic methods

Electropherograms were edited in the program BioEdit (Hall, 2005), and sequences were aligned using default parameters in ClustalW (Chenna et al., 2003). For the 28S gene, all sequences were used, including those from the outgroup family Pseudodiaptomidae. The centropagid lineages found to form the sister group to all other centropagids in the 28S tree were used to root the 16S phylogeny, allowing the alignment of this faster-evolving gene to be performed only using the ingroup taxa (thus reducing the number of gaps and alignment ambiguities). This approach increased our confidence in the alignments, as there were few resulting gaps in alignments for both genes (see Section 3).

A chi-square test was performed in PAUP* version 4b10 (Swofford, 1998) for each gene to test for variation in nucleotide composition among taxa. The program Modeltest 3.06 (Posada and Crandall, 1998) was used to select the best model of nucleotide substitution, for comparison with the results from MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Kimura 2-parameter (K2P) (Kimura, 1980) distances were also calculated in MEGA3 (Kumar et al., 2004) to assess levels of intraspecific and interspecific variation, because employing a commonly-used distance measure facilitates comparisons across taxonomic groups and across studies.

Phylogenies were first inferred for each gene separately using Bayesian analysis in MrBayes 3.0. *Pseudodiaptomus inopinus* was used as the outgroup for the 28S phylogeny, while *Centropages* spp. were used to root the 16S analysis. All model parameters were estimated by MrBayes, and the Monte Carlo Markov chain (MCMC) analysis was run for 1,100,000 generations, with four chains (one cold, three hot) and trees sampled every 100 generations. The first 1000 trees were discarded as the “burn-in” phase, and the remaining 10,000 were used to construct a 50% majority-rule consensus tree with all compatible nodes included. Nodal support values (posterior probabilities) were calculated by MrBayes as the percentage of sampled trees containing that node. Two separate Bayesian analyses were performed to verify that the same consensus phylogenies were obtained.

After conducting a partition homogeneity test in PAUP (with settings: heuristic search, nearest-neighbour exchange, 100,000 replicates, 1000 trees held per replicate with autoincrease set to 100, random sequence addition), a combined 16S + 28S Bayesian analysis was performed for those taxa sequenced for both genes, with separate (unlinked) models estimated for each gene.

Since sequences were of different lengths, ambiguous bases (Ns) were added to the beginning or end of some sequences during alignment. Although MrBayes ignores sites that contain alignment gaps in any sequence, such as those introduced to maintain positional homology in ribosomal genes, it uses information from sites that merely contain missing data in a subset of sequences. The effect of including these sites containing missing data was examined by repeating all analyses excluding shorter sequences and includ-

Table 1

List of Centropagidae species included in this study. Country and major regional codes are: ANT – Antarctica, ARG – Argentina, AUS – Australia, BOL – Bolivia, BRAZ – Brazil, CAI – circumantarctic islands, CAM – Central America, CAN – Canada, CHILE, CHN – China, COL – Colombia, ECU – Ecuador, JPN – Japan, NZ – New Zealand, PER – Peru, TAS – Tasmania, UR – Uruguay, USA – United States of America. The countries/regions highlighted in bold are those from which samples were obtained. Additional abbreviations used in the locality column are: SWA – south-western Australia, WA – Western Australia, na – not available, and em dash – same as site listed above. Habitats are as follows: E – estuarine, L – permanent lakes, M – marine, R – rivers and streams, S – saline lakes (mostly temporary), T – temporary (non-saline) ponds and lakes.

Species	Countries inhabited by each species	Habitat of each species	Specimen code	Locality sampled	16S GenBank	28S GenBank
South American Boeckella						
<i>B. antiqua</i> Menu-Marque and Balseiro 2000	ARG	T, L	084	Type locality: Laguna los Juncos, Río Negro (41° 03.550' S, 71° 00.406' W)	GU049679	GU049733
<i>B. antiqua</i>			085	–	GU049680	na
<i>B. bergi</i> Richard 1897	ARG, BRAZ, CHILE?, UR	R, T	003	Pool at Arroyo de la Ventana, Río Negro (41° 45.836' S, 66° 30.113' W)	na	GU049734
<i>B. bergi</i>			004	–	GU049681	GU049735
<i>B. brasiliensis</i> (Lubbock 1855)	ARG, CHILE	L, T	011	Pool by Provincial Route 41, Santa Cruz (46° 50.936' S, 71° 52.293' W)	GU049682	GU049736
<i>B. brasiliensis</i>			012	–	GU049683	na
<i>B. gracilipes</i> Daday 1901	ARG, CHILE	L, T	070	Laguna de los Horcones, Mendoza (32° 48.344' S, 69° 56.607' W)	GU049684	na
<i>B. gracilis</i> (Daday, 1902)	ARG, BOL, CHILE, COL, ECU, PER	T, L	E234	Laguna Salada Grande, Buenos Aires province (36° 57.017' S, 56° 58.789' W)	na	GU049737
<i>B. meteoris</i> Kiefer 1928	ARG, BOL, CHILE		005	Laguna Bombilla, Provincial Route 24, Chubut (44° 08.749' S, 69° 15.692' W)	GU049685	GU049738
<i>B. meteoris</i>			006	–	GU049686	GU049739
<i>B. meteoris</i>			E679	km 2193, Rt. #3, Santa Cruz (48° 48.713' S, 67° 40.937' W)	na	GU049740
<i>B. michaelseni</i> (Mrazek 1901) (lineage A)	ARG, CHILE, CAI	L, T	009	Pool in swamp, Provincial Route 41, Santa Cruz (46° 50.936' S, 71° 52.293' W)	GU049687	GU049741
<i>B. michaelseni</i> (lineage A)			010	–	GU049688	GU049742
<i>B. michaelseni</i> (lineage B)			091	km 2923, Rt. #3, Tierra del Fuego (54° 28.400' S, 67° 14.000' W)	GU049689	GU049743
<i>B. michaelseni</i> (lineage B)			092	–	GU049690	GU049744
<i>B. michaelseni</i> (lineage B)			E669	–	na	GU049745
<i>B. poopoenis</i> Marsh 1906	ARG, BOL, CHILE, PER	S	015	Pool near Sarmiento, Chubut (approx. 45° 40' S, 68° 60' W)	GU049691	GU049746
<i>B. poopoenis</i>			016	–	GU049692	GU049747
<i>B. poopoenis</i>			E233	Laguna de los Horcones, prov. Buenos Aires (37° 3.804' S, 57° 0.185' W)	na	GU049748
<i>B. poppei</i> Mrazek 1901	ANT, ARG, CHILE, CAI	L, T	065	Along Rt. #3, Tierra del Fuego (morphological type "B") (54° 09' S, 67° 15' W)	GU049693	na
<i>B. poppei</i>			069	Pond by Rt. #3 Santa Cruz (49° 14.994' S, 67° 46.726' W)	GU049694	GU049749
<i>B. schwabei</i> Brehm 1937	ARG, CHILE	T	007	Pool near Dina Huapi, Río Negro (41° 5.341' S, 71° 9.452' W)	GU049695	GU049750
<i>B. schwabei</i>			008	–	GU049696	GU049751
<i>B. titicacae</i> Harding 1955	BOL, CHILE, PER	L	079	Lake Titicaca (16° 12.857' S, 68° 41.434' W)	GU049697	na
Australasian Boeckella						
<i>B. bispinosa</i> Bayly 1967	AUS, TAS	T	bsp1	Goonapping Swamp, near Brookton, SWA (32° 9.033' S, 116° 35.683' E)	na	GU049752
<i>B. bispinosa</i>			039	–	GU049698	na
<i>B. fluvialis</i> Henry 1922	AUS	R*	flv1	Murray River billabong, southern New South Wales (approx. 36° S, 147° E)		GU049753
<i>B. hamata</i> Brehm 1928	NZ	L	019	Tomahawk Lagoon, South Island (45° 53.990' S, 170° 32.565' E)	GU049699	na
<i>B. hamata</i>			020	–	GU049700	na
<i>B. robusta</i> Sars 189	AUS	T	rob1	Kulicup Swamp, west of Kojonup, SWA (33° 49.650' S, 116° 40.217' E)	na	GU049754
<i>B. robusta</i>			024	Tuckers Rd. Melaleuca Swamp SWA (34° 24.900' S, 117° 14.880' E)	GU049701	na
<i>B. triarticulata</i> (Thomson, 1883)	AUS, NZ, TAS	L, T	tri1	Lake Logue, west of Eneabba, SWA (29° 51.333' S, 115° 8.833' E)	na	GU049755
<i>B. triarticulata</i>			049	–	GU049702	na
<i>B. triarticulata</i>			tri3	Coomalbidgup Swamp, west of Esperance, SWA (33° 43.133' S, 121° 22.217' E)	na	GU049756
<i>B. triarticulata</i>			043	–	GU049703	na
<i>B. triarticulata</i>			044	–	GU049704	na
<i>B. triarticulata</i>			051	Shark Lake, Esperance, SWA (33° 46.117' S, 121° 51.667' E)	GU049705	na
Calamoecia + Parabroteas clade						
<i>C. ampulla</i> (Searle 1911)	AUS, NZ, TAS	L, T	amp1	Coomalbidgup Swamp, west of Esperance, SWA (33° 43.133' S, 121° 22.217' E)	na	GU049757
<i>C. ampulla</i>			053	–	GU049706	na
<i>C. ampulla</i>			054	–	GU049707	na
<i>C. ampulla</i>			amp2	Shark Lake, Esperance, SWA (33° 46.117' S, 121° 51.667' E)	na	GU049758

(continued on next page)

Table 1 (continued)

Species	Countries inhabited by each species	Habitat of each species	Specimen code	Locality sampled	16S GenBank	28S GenBank
<i>C. ampulla</i>			057	–	GU049708	na
<i>C. ampulla</i>			058	–	GU049709	na
<i>C. attenuata</i> (Fairbridge 1945)	AUS	L, T	045	Goonapping Swamp, near Brookton, SWA (32° 9.033' S, 116° 35.683' E)	GU049710	na
<i>C. attenuata</i>			046	–	GU049711	na
<i>C. attenuata</i>			att3	Kulicup Swamp, west of Kojonup, SWA (33° 49.650' S, 116° 40.217' E)	na	GU049761
<i>C. attenuata</i>			055	–	GU049714	na
<i>C. attenuata</i>			056	–	GU049715	na
<i>C. attenuata</i>			047	Shark Lake, Esperance, SWA (33° 46.117'S, 121° 51.667'E)	GU049712	na
<i>C. attenuata</i>			048	–	GU049713	na
<i>C. attenuata</i>			att2	–	na	GU049760
<i>C. attenuata</i>			att1	–	na	GU049759
<i>C. canberra</i> Bayly 1962	AUS	T	086	Wooramel canegrass pan, south of Carnarvon, mid-WA (25° 40.866' S, 114° 13.236' E)	GU049716	na
<i>C. canberra</i>			087	–	GU049717	na
<i>C. clitellata</i> Bayly 1962	AUS	S	cli1	North Parriup Lake, east of Hopetoun, SWA (33° 51.983'S, 120° 38.217'E)	na	GU049762
<i>C. clitellata</i>			041	–	GU049718	na
<i>C. clitellata</i>			042	–	GU049719	na
<i>C. halsei</i> Bayly 1998	AUS	S	029	Wooramel canegrass pan, south of Carnarvon, mid-WA (25° 40.866' S, 114° 13.236' E)	GU049720	na
<i>C. halsei</i>			030	–	GU049721	na
<i>C. tasmanica subattenuata</i> (Fairbridge 1945) (lineage A)	AUS, NZ, TAS	T, L	tas1	Lake Joondalup, Perth, SWA (approx. 31° 46'S, 115° 48'E)	na	GU049763
<i>C. tasmanica subattenuata</i> (lineage A)			059	–	GU049722	GU049764
<i>C. tasmanica subattenuata</i> (lineage A)			060	–	GU049723	na
<i>C. tasmanica subattenuata</i> (lineage B)		T	037	Kulicup Swamp, west of Kojonup, SWA (33° 49.650' S, 116° 40.217' E)	GU049724	na
<i>C. tasmanica subattenuata</i> (lineage B)			tas2	–	na	GU049765
<i>P. sarsi</i> (Daday, 1901)	ARG, CHILE, CAI	T, L	017	Pond in swamp, western Chubut (44° 02.787' S, 71° 27.309' W)	GU049725	na
<i>P. sarsi</i>			018	–	GU049726	GU049766
<i>P. sarsi</i>			E692	Ditch, Rt. #3, just S of Río Chico, Santa Cruz	na	GU049767
<i>P. sarsi</i>			E711	Pond, Rt. #46, 66 km N of Tres Lagos, Santa Cruz	na	GU049768
Other Centropagidae						
<i>Centropages bradyi</i> Wheeler 1900	Widespread oceanic	M		California Current, CALCOFI station (from Goetze (2003))	AY335895	na
<i>Centropages furcatus</i> (Dana 1849)	Widespread oceanic	M	073	Mouth of Fort Pierce Inlet, Florida (27° 28' 18" N, 80° 17' 20" W)	GU049727	na
<i>Centropages furcatus</i>			074	Offshore, Florida (27° 31' 34" N, 79° 58' 29" W)	GU049728	na
<i>Centropages furcatus</i>			075	–	GU049729	GU049769
<i>Gladioferens imparipes</i> Thomson 1946	AUS	E	088	Swan Estuary, WA (approx. 32° 3' S, 115° 44' E)	na	GU049770
<i>Gladioferens imparipes</i>			089	–	na	GU049771
<i>Gladioferens imparipes</i>			E1670	–	na	GU049772
<i>Limnocalanus johanseni</i> Marsh 1920	CAN (and Alaska, USA)	L, E, M	E1648	Tuktoyaktuk, NWT (approx. 69° 24' N, 133° 0' W)	na	GU049773
<i>Limnocalanus macrurus</i> Sars 1863	Circumarctic	L, E, M	E1645	Lake Ontario	na	GU049774
<i>Osphranticum labronectum</i> Forbes 1882	USA, CAM	T, L	071	Maryland (38° 56' 30" N, 77° 7' 0" W)	GU049730	GU049775
<i>Osphranticum labronectum</i>			072	–	na	GU049776
<i>Sinocalanus sinensis</i> (Poppe 1889)	JPN, CHN	E	031	JPN-ROK. Rokkaku River estuary, Ariake Sound, Kyushu (33° 11' 37" N, 130° 12' 40" E)	GU049731	na
<i>Sinocalanus sinensis</i>			032	–	GU049732	na
Outgroup						
<i>Pseudodiaptomus inopinatus</i> Burckhardt 1913		E	033	JPN-ROK. Rokkaku River estuary, Ariake Sound, Kyushu (33° 11' 37" N, 130° 12' 40" E)	na	GU049777
<i>P. inopinatus</i>			033a	–	na	GU049778
<i>P. inopinatus</i>			034	–	na	GU049779

* Wetlands; floodplains; still or low-flowing river pools.

ing only those sites having data for all sequences, resulting in alignments of 407 bp containing 50 sequences for 16S and 669 bp with 36 sequences for 28S. Results were very similar to those obtained using the long alignments, and were identical in all major aspects discussed in this study, and thus are not treated further. The larger datasets better demonstrate species relationships and intraspecific variability.

2.4. Biogeography and habitat shifts

Prior to biogeographic analysis, we generated a single best hypothesis of molecular phylogenetic relationships that was as comprehensive as possible. Since slightly different sets of taxa amplified successfully using the 16S and 28S primers, deeper phylogenetic relationships were based upon a composite, consensus topology, which was compatible with the results from each gene analyzed alone. Relationships within the shallower clade consisting of the genera *Calamoecia*, *Parabroteas*, and *Boeckella* were taken from the faster-evolving 16S fragment; two species in this group lacking 16S were omitted. Subsequently, biogeographic regions and habitats were plotted onto this phylogeny using the maximum parsimony criterion in the program *Mesquite* version 1.11 (Maddison and Maddison, 2006). The parsimony criterion was selected (rather than ML methods) for the biogeographic reconstruction because this method is expected to perform well when change is relatively rare (up to about 0.3 transitions per branch) (Maddison and Maddison, 2000); moreover, biogeographic shifts are not expected to exhibit equal likelihood of occurrence along different branches of the phylogeny.

Regions were broadly defined as northern hemisphere, South America, and Australasia, to explore the hypothesized Gondwanaland origins of the southern inland species. To estimate the number of independent colonizations of inland waters, genetic evidence was jointly considered along with finer-scale knowledge of distributions and habitat occupancy. Transitions amongst types of aquatic environments were examined based upon the categories: M – marine, E – estuarine, T – temporary or seasonal ponds and lagoons, L – permanent lakes and ponds, R – rivers, and S – athalassic saline environments. The distributions and primary habitats of species were obtained from Jolly (1957), Ringuet (1958), Bayly (1992a–c), Maly and Maly (1997), Maly et al. (1997), and observations by the authors. A few species exhibit multiple habitat types (see Fig. 4); since polymorphisms are not permitted in this analysis, the primary habitat type for each species was selected for the analysis.

Two nodes represented a putative case of parallel vicariant speciation between the Australian and South American continents. 16S was used to test for concordant divergence times between these nodes, because this fragment contained far more information (>5-fold greater divergence) and phylogenetic resolution at this level than 28S. A likelihood ratio test was performed to detect departures from clock-like behaviour in molecular evolutionary rates. The 16S Bayesian phylogeny and parameter estimates (post burn-in) were entered into PAUP; and likelihood scores were calculated for the tree with and without a molecular clock enforced. Being a comparison of nested hypotheses, the likelihood ratio statistic (twice the difference in $-\ln$ Likelihood scores between the models) is expected to follow a chi-square distribution, with the degrees of freedom equal to the difference in number of parameters (number of sequences minus 2) (Felsenstein, 1981).

Upon rejecting the strict molecular clock, a relaxed-clock model (uncorrelated exponential model; see Drummond et al., 2006) was used to estimate the divergence times at the two nodes of interest, via Bayesian analysis conducted in the program *BEAST* version 1.4 (Drummond and Rambaut, 2006). A strict-clock analysis was performed for comparison, as clock models can sometimes perform well even when a clock is rejected (Drummond et al., 2006). Be-

cause of a lack of appropriate taxon-specific calibration points, the estimation of absolute divergence times was precluded here. Therefore, relative divergence times, in arbitrary units, were estimated instead, implementing a fixed mutation rate of 1.0. The MCMC analysis was run in *BEAST* for 10,000,000 generations with trees sampled every 1000 and the first 10% discarded as burnin. Mean divergence times and 95% confidence intervals (C.I.) were summarized using the program *Tracer* version 1.3 (Rambaut and Drummond, 2005). Non-overlapping confidence intervals were interpreted as evidence of non-concordance, while overlapping intervals indicated potential concordance. However, a null model (e.g. see Crisp and Cook, 2007) would have been needed to provide a statistical test of congruence that provided a *p*-value, and thus this assessment is preliminary.

3. Results

3.1. Relationships and patterns of genetic diversity

The final 16S alignment contained 55 sequences and was 416 bp in length, of which 359 positions contained no gaps, while 28S consisted of 46 sequences aligned to a total of 971 positions, of which 929 contained no gaps. Nucleotide composition was strongly A-T biased in 16S (38.01% A, 9.88% C, 12.25% G, 39.86% T) but not 28S (21.73% A, 32.73% C, 23.79% G, 21.76% T). However, composition was relatively homogeneous across taxa for each gene (16S: 55 sequences, $\chi^2 = 91.34$, d.f. = 162, $P = 1.00$; 28S: 48 sequences, $\chi^2 = 17.78$, d.f. = 141, $P = 1.00$). For both genes, the results of *Modeltest* supported the inclusion of parameters modeling variation in substitution rate among sites (gamma parameter) and accounting for the proportion of invariant sites (TVM+I+G for 16S; TrV+I+G model for 28S).

Both the 28S (Fig. 2) and 16S (Fig. 3) Bayesian analyses reconstructed a monophyletic clade containing the southern-hemisphere continental genera *Boeckella*, *Calamoecia*, and *Parabroteas* with 99–100% posterior probability. *Gladioferens imparipes*, an Australian estuarine species, was recovered as the sister group to this clade in the 28S tree. However, its placement had just 81% posterior probability support and this taxon was not successfully amplified for 16S. Together, the two trees revealed that all of the northern genera that are primarily continental or estuarine (*Sinocalanus*, *Limnocalanus*, *Osphranticum*) were more closely related to the marine genus *Centropages* than to the southern continental group.

The genus *Boeckella* was recovered as monophyletic in both analyses, but this topology was only supported by 28S (100% posterior probability in 28S phylogeny, 44% in 16S analysis). 28S showed little resolution among species within this group, while distinctive South American and Australasian clades were recovered by 16S (80% and 100% support, respectively). Also in both trees, the genera *Calamoecia* and *Parabroteas* together formed a clade (50% posterior probability 28S, 97% for 16S), but *Parabroteas* was found in both analyses to be nested within a paraphyletic *Calamoecia*. 16S provided much more phylogenetic resolution at the species level than did 28S. However, a few nodes remained poorly resolved or poorly supported among species within the genus *Boeckella*.

The partition homogeneity hypothesis was not rejected ($P = 0.12$) and so a combined 16S + 28S analysis was also performed. Although it contained fewer taxa ($n = 24$) than either separate analysis, the combined tree (not shown) confirmed the primary patterns of a monophyletic *Boeckella* + *Parabroteas* + *Calamoecia* clade, a monophyletic *Boeckella* divided into South American and Australasian sister clades, and *Parabroteas* nested within a paraphyletic *Calamoecia*.

Eight of the 11 centropagid species having multiple 28S sequences were recovered as being monophyletic (Fig. 2), the excep-

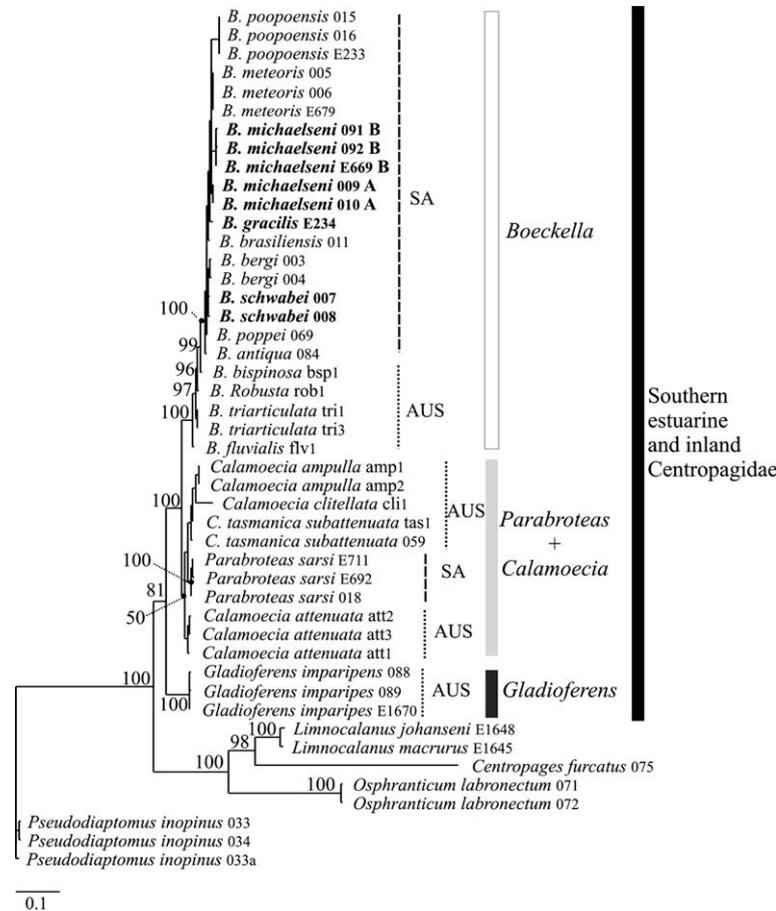


Fig. 2. Bayesian consensus tree for members of the family Centropagidae based on 28S sequences. Posterior probability values are given to the left of select nodes. Bold highlighting is used to designate those individuals whose species status requires clarification; these are addressed in the text. Geographical localities are: AUS – Australasia; SA – South America.

tions involving a paraphyletic topology (*Boeckella schwabei*, *B. michaelseni*, and *Calamoecia tasmanica subattenuata*). Typically, there was little genetic divergence within species. Maximum intra-specific K2P distances were small in 28S, generally up to 0.2% within *Boeckella* species and up to 0.4% within species in the clade containing the genera *Calamoecia* and *Parabroteas* (Table 2). However, the nominal species *B. michaelseni* contained two groups separated by an average distance of 0.8%. The two sequences of *C. tasmanica subattenuata* included in this 28S analysis were similar to one another and displayed a shallow paraphyletic topology. However, another sequence of this species (excluded from the Bayesian phylogeny due to its short length, 540 bp) showed 4.0% 28S divergence from its supposed conspecifics and is designated as lineage B in Table 1. The separation between these two lineages of this morphospecies can be clearly observed in the 16S tree (Fig. 3). The splits within both *B. michaelseni* and *C. tasmanica subattenuata* involve distances that were more typical of interspecific comparisons, which ranged from 0.2% to 3.3% in *Boeckella* and from 1.1% to 5.6% in *Calamoecia* and *Parabroteas*. *Boeckella schwabei* and *B. gracilis*, considered synonyms by some authors, showed 1.0% 28S divergence from one another, which is within the range of typical interspecific divergences found for *Boeckella*.

Sixteen of 18 species having multiple 16S sequences were found to be monophyletic (Fig. 3), the exceptions being *B. poppei*, which was previously found to be monophyletic using COI (Adamowicz et al., 2007), and *C. tasmanica subattenuata*. Mean interspecific 16S distances ranged from 4.0% to 18.6% among *Boeckella* species and 10.3–16.0% among members of the *Calamoecia*/*Parabroteas* clade, while maximum intraspecific divergences were generally

0–2.8% and 0–3.5% for these two clades. Again, *B. michaelseni* formed two divergent sister clades (7.6% 16S distance). The two lineages of *C. tasmanica subattenuata* were highly divergent (16.0% 16S distance), and these were not sister lineages, one instead grouping more closely with *C. clitellata*. *Boeckella titicacae* and *B. gracilipes*, sometimes considered synonyms, showed 4.9% 16S divergence, within the range of other interspecific divergences observed.

3.2. History of the Centropagidae

All of the southern hemisphere estuarine and continental species were part of a single monophyletic group, but there were two cases of splits between Australasian and South American taxa (Fig. 4A). According to the 16S gene, the split between the two clades of *Boeckella* was older than the *Parabroteas*-*Calamoecia* divergence by about 1.8-fold (Table 3). The 95% confidence intervals on the relative dates of these nodes, based upon the relaxed-clock analysis, were nearly non-overlapping (0.068–0.131 vs. 0.127–0.224, in arbitrary units), suggesting non-concordant divergence. There was no overlap in confidence intervals from the strict-clock analysis (not shown).

Molecular phylogenetic results indicate a single continental invasion for the southern centropagids (Fig. 4A). Due to their distributions on different continents, and phylogenetic position in relationship to *Centropages* (which, being marine, possesses what is presumed to be the ancestral habitat based upon comparisons with calanoids more broadly), *Osphranticum* (southeastern North America), *Sinocalanus* (east Asia), and *Limnocalanus* (northern Holarctic)

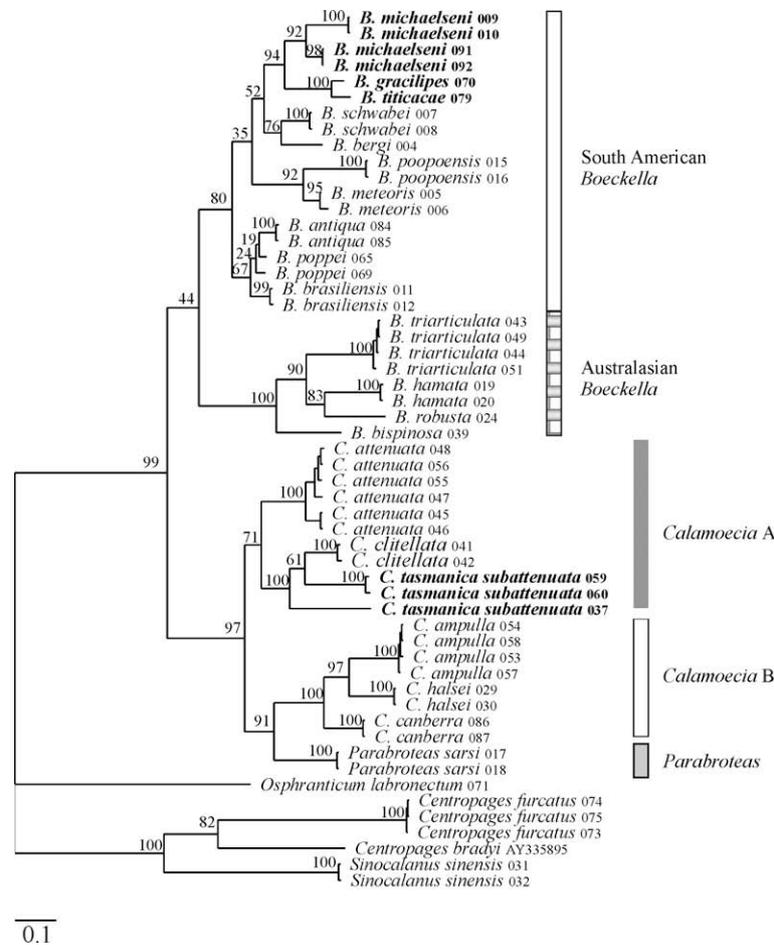


Fig. 3. Bayesian 16S tree for centropagid species, with major clades indicated and posterior probabilities given for all nodes at or above the species level. Bold highlighting is used to designate those individuals whose species status requires clarification; these are addressed in the text, and key divergence values are provided in Table 2.

Table 2

Summary of key genetic distances (K2P) in the Centropagidae. The sample size of species includes two putative cryptic species within *B. michaelsoni* and two within *C. tasmanica subattenuata*. Divergence values that may have taxonomic implications are indicated in the right column; these are discussed in the text and indicated in bold in Figs. 2 and 3.

Group	Maximum intraspecific divergences	Mean interspecific divergences	Notable divergences
<i>Comparisons within and among species</i> <i>Boeckella</i> species ($n = 15$)	0–2.8% 16S	4.0–18.6% 16S	<i>B. michaelsoni</i> A/B: 7.6% 16S
	0.0–0.2% 28S	0.2–3.3% 28S	<i>B. titicacae</i> / <i>B. gracilipes</i> : 4.9% 16S
			<i>B. schwabei</i> / <i>B. gracilis</i> : 1.0% 28S
<i>Calamoecia</i> + <i>Parabroteas</i> species ($n = 8$)	0.0–3.5% 16S	10.3–16.0% 16S	<i>C. tasmanica subattenuata</i> A/B: 16.0% 16S
	0.0–0.4% 28S	1.1–5.6% 28S	<i>C. tasmanica subattenuata</i> A/B: 4.0% 28S*

* The divergent sequence of *C. tasmanica subattenuata* (lineage B) is not included in Fig. 2 because of its short length (540 bp).

all apparently represent independent invasions of continental waters. Both *L. macrurus* and *L. johanseni* are euryhaline and can be found in both fresh and coastal marine waters, suggesting that each made the inland shift separately.

Once inland, centropagids continued to undergo habitat shifting, with multiple independent transitions observed (Fig. 4B). Among the 23 species of *Boeckella*, *Parabroteas*, and *Calamoecia* included in the 16S tree, seven shifts are reconstructed, with habitats being divided into lakes, temporary ponds, rivers, and saline lakes. Both of the species inhabiting inland saline lakes included in our dataset (*Boeckella poopoenis* and *Calamoecia clitellata*) occurred in nested phylogenetic positions within their respective freshwater genera.

4. Discussion

The molecular phylogenetic results presented here answer several unresolved questions regarding the complex biogeographic history of the Centropagidae. It is clear that multiple continental invasions from the marine realm have been key determinants of the diversity and distribution patterns in the family. As our results strongly indicate the monophyly of the large group of species inhabiting the southern continents, Boxshall and Jaume's (2000) hypothesis of five independent invasions is supported. Given this propensity for continental colonization, which is one of the highest among copepod families, the large temporal separation between the earliest (likely Cretaceous) case and the four recent ones is puzzling.

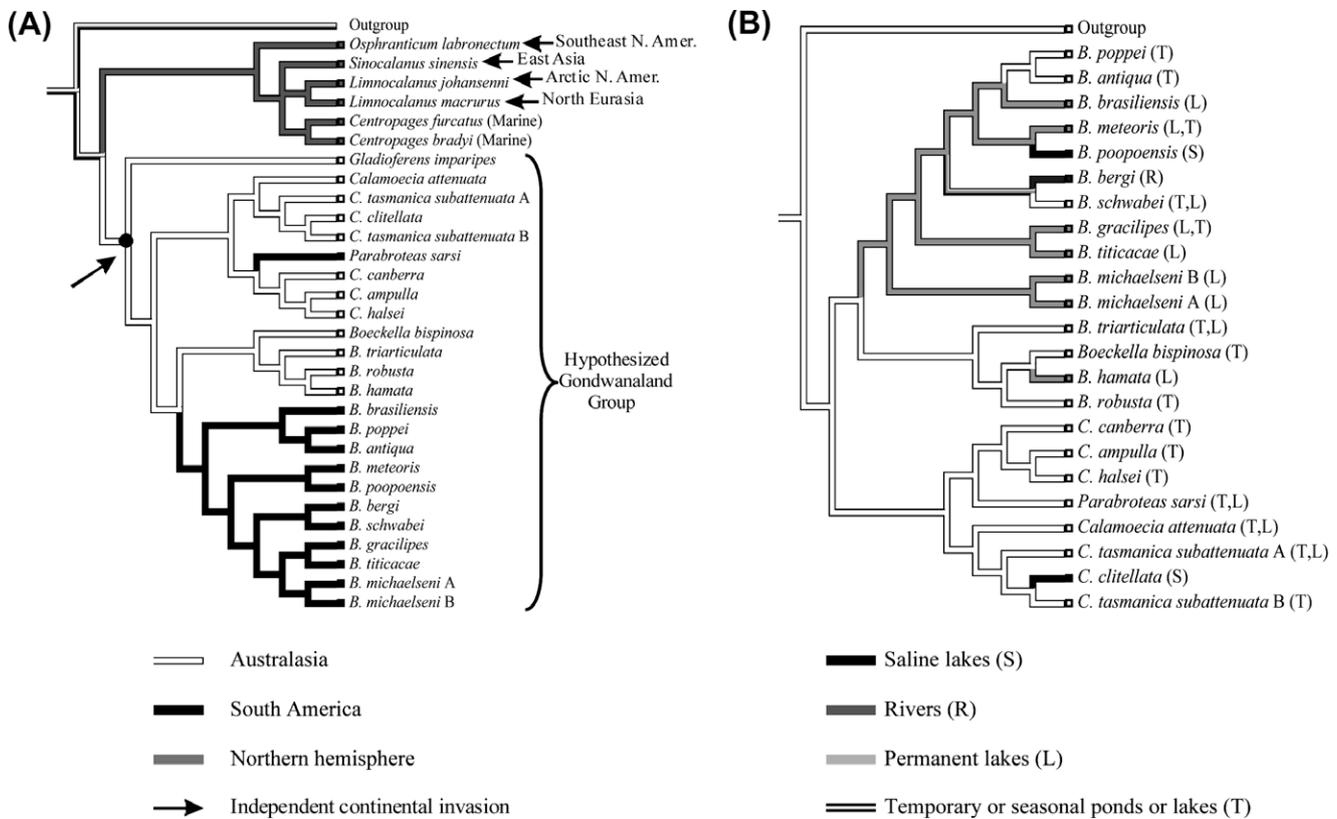


Fig. 4. Biogeographic and habitat shifts in the Centropagidae, reconstructed using the maximum parsimony (MP) criterion. The phylogenetic hypothesis was based upon a composite consensus of 28S (Fig. 2) and 16S (Fig. 3) results for generic-level relationships and 16S for species-level relationships. (A) Shifts in biogeographic regions, showing the monophyly of the southern-hemisphere continental species and two cases of Australasia–South America splits. Phylogeographic evidence (see text) supports at least five independent cases of continental invasion. (B) Shifts among continental habitat types among *Boeckella*, *Calamoecia*, and *Parabroteas* species.

Table 3
Relative divergence times for two nodes showing putative South America/Australasia vicariance.

	Australasian vs. South American <i>Boeckella</i>	<i>Parabroteas</i> vs. sister <i>Calamoecia</i> A clade	Concordant divergence?
<i>Comparing two divergences: parallel vicariant cladogenesis?</i>			
16S divergence (K2P)	17.9%	11.9%	
Relative divergence time (in arbitrary units) based upon Bayesian analysis of 16S sequences	Mean: 0.175 (95% C.I.: 0.127–0.224)	Mean: 0.09742 (95% C.I.: 0.06791–0.131)	<i>Boeckella</i> split was estimated as 1.8-fold older than the <i>Parabroteas</i> / <i>Calamoecia</i> A node, based on Bayesian analysis. However, the 95% confidence intervals overlap slightly.

zling and is discussed further below. Other types of habitat shifts, such as those between ponds and lakes, have also been important in the diversification of the centropagids. The molecular evidence shows that species inhabiting inland saline lakes belong to freshwater clades (supporting Bayly, 1964), and likely do not trace their ancestry directly to marine waters or epicontinental seas, thus refuting Maly (1996). Both repeated invasion from the sea and *in situ* diversification in continental waters (via vicariance as well as ecological shifting) are important in structuring freshwater diversity patterns in this interesting group of copepods.

4.1. History of continental invasions

The ancient supercontinent of Gondwanaland apparently sustained just a single centropagid invasion, which diversified into a large species group that still primarily inhabits southern continental waters, including members of the genera *Boeckella*, *Parabroteas*, *Calamoecia*, and *Gladioferens*. Although an appropriate calibration point is lacking for a molecular clock analysis, the large depths of

genetic divergence are consistent with an ancient event. Biogeographic and geological evidence suggests that this invasion occurred sometime after the breakup of Pangaea into Laurasia and Gondwanaland, which was initiated ~185 MYA with the opening of the Central Atlantic (Veevers, 2004), but before the final partitioning and dispersal of the continents of Gondwanaland. Boxshall and Jaume (2000) suggested that the invasion occurred prior to the split between East and West Gondwana, which they suggested to be ~120 MYA (although dispersal links could have been interrupted somewhat earlier because the opening of the Indian Ocean commenced 150–156 MYA (Veevers, 2004)). They attributed the absence of centropagids from Africa to either their exclusive occupancy of southern Gondwanaland or to their later extinction, which could have been facilitated by the general high level of African–Laurasian faunal exchanges (Gheerbrant and Rage, 2006). However, this absence could also be explained if the colonization occurred in another part of Gondwanaland after Africa became separated from all three of the other southern continents, ~105 MYA (McLoughlin, 2001). The more recent connections between South

America and Australia via a mild Antarctica might have permitted dispersal until ~30 MYA (McLoughlin, 2001; Knapp et al., 2005), which could be interpreted as the upper threshold of the potential timeframe of the colonization. Thus, a Cretaceous invasion seems most probable, but a colonization as recent as the Oligocene is plausible.

While a single large southern clade is supported here, future molecular work is desirable to confirm that the New Caledonian genus *Parathalassius*, the Australasian *Hemiboeckella*, and South American *Neoboeckella* were part of this clade. Additionally, molecular evidence should be used to test whether the sole northern record for any of these southern genera (*Boeckella triarticulata* in Mongolia) also represents a case of Gondwanan vicariance, or whether anthropogenic introduction could be the cause of this disjunction.

Our results provide further insights into the early inland history of the centropagids that were not available without the molecular data. Members of this southern continental clade were evidently both widespread and already diversified in at least some parts of Gondwanaland at the time of the separation of Australia and South America. The topology taken alone is suggestive of parallel vicariant cladogenesis in two separate lineages: within the genus *Boeckella* and between *Parabroteas* and part of *Calamoecia*. However, the relaxed-clock analysis indicated that the relative divergence times are not concordant, with mean times differing 1.8-fold and with their 95% C.I.'s overlapping only slightly. It is possible that one vicariant and one dispersal event were involved in these distributions; it is not possible to tell which is which in the absence of a calibration point providing an absolute rather than relative timescale. Both types of cladogenesis are indicated for members of the tree genus *Nothofagus* (southern beech) (Knapp et al., 2005), even though trans-oceanic dispersal had previously been ruled out for this group due to presumed poor dispersal ability. Another possibility is that different geological events, such as the East–West Gondwana split and the final sundering of South America from Antarctica and Australia, were involved but that both are cases of vicariance. However, to investigate these possibilities and to reject congruent cladogenesis more definitively, additional data are needed to overcome the variance of molecular evolutionary rates and to include species inhabiting the smaller fragments of Gondwanaland.

Combined distributional and phylogenetic evidence indicates that at least four more inland invasions occurred within the Centropagidae. Even though phylogenetic relationships remain to be studied within the genus *Sinocalanus*, the generic affinities and distributions demonstrate at least one independent invasion within this genus. Boxshall and Jaume (2000) categorized this genus as a case of “colonization” (invasion followed by speciation) and suggested that this occurred in the “post-glacial” period. However, much of the range of *Sinocalanus* species occurs in regions south of those covered by glaciers, and the depths of divergence among species in this group are required to test timeframes for this event. *Osphranticum labronectum* belongs to a monotypic inland genus and is thus suspected to represent a relatively recent invasion, but further sampling of marine centropagid lineages will be required to investigate its history. *Limnocalanus macrurus* and *L. johanseni*, although primarily distributed in freshwaters, are euryhaline and can be found in both coastal and inland settings, suggesting that freshwater invasion occurred quite recently in each of these taxa. A phylogeographic study on *Limnocalanus* (Dooh et al., 2006) confirmed a close relationship between *L. macrurus* populations living in inland North American lakes and those inhabiting both a marine zone (Beaufort Sea) and coastal arctic lakes, which represent populations recently captured from the sea via isostatic rebound. This pattern affirms a Pleistocene-era invasion. Moreover, *L. macrurus* likely represents multiple independent inva-

sions, due to its presence in lakes in Europe, Greenland, and northern North America; the close genetic relationship between Baltic and North American sequences definitively rules out vicariance as an explanation for this multicontinental distribution. As *L. johanseni* is quite distantly related to *L. macrurus* (Dooh et al., 2006), with >20% COI divergence between them, it also represents an independent invasion rather than a recent offshoot of the young inland populations of *L. macrurus*.

Interestingly, these four cases of recent continental colonization/incursion are much more recent than inferred for the *Boeckella* group of genera. It is not clear why this would be so. Centropagids seem to move inland readily, due perhaps to their evolutionary euryhalinity and ability to produce resting eggs. Thus, one might expect phylogenetic evidence of inland invasion at various points in time between the Cretaceous and Pleistocene. Although more colonizations may be revealed through additional taxonomic sampling, we suspect that our coverage was widespread enough to uncover most cases. One possibility to explain this gap is that inland invasions did indeed occur in the intermediate time period but that there remains no evidence in the form of extant representatives. For example, centropagids may be out-competed by other lineages. Bayly (1992a, 1995; and see Boxshall and Jaume, 2000) suggested that freshwater diaptomids replaced the centropagids in South America in all areas except at high latitudes and altitudes following the closing of the Isthmus of Panama. Such replacements could have occurred at various times in the past as well, and would be undetected due to the lack of a fossil record. A second possibility, not mutually exclusive from the first, is that centropagids are particularly adept at colonizing newly-available and extreme environments, such as created upon the retreat of major ice sheets. As such, the inland invasion rate may have truly increased during the Pleistocene. Finally, the Pleistocene glaciers may have been physically responsible for moving coastal lineages inland via the lakes created at the margins of ice sheets. As such, this relatively unusual period in geological history may have been instrumental in some of the invasions. In particular, this may have affected euryhaline arctic marine species such as *Limnocalanus* spp., whose inland distribution is closely connected with glacial history (Dadswell, 1974; Dooh et al., 2006). However, this mechanism cannot readily explain inland invasions in more sub-tropical or temperate settings, such as the cases of *Osphranticum* and *Sinocalanus*. Thus, a combination of factors may have been involved in determining the temporal distribution of centropagid continental invasions.

4.2. Ancient invasion: continuing habitat shifts inland

Several forms of habitat shifts have been part of the evolutionary history of the Centropagidae. This has involved very broad shifts from marine to continental settings, as well as shifts among different types of inland water bodies.

Parts of Bayly's (1964) hypothesis regarding the directions of large-scale shifts (i.e. marine to estuarine to fresh to inland saline waters) have received support here, while others await further phylogenetic analysis based upon more complete taxonomic sampling. First of all, transitions from marine or estuarine to continental environments appear to have been unidirectional in the centropagids, based upon current biogeographic and phylogenetic evidence. Similarly, the last shift in Bayly's sequence is also well supported. In particular, both species in our dataset inhabiting saline lakes (*Calamoecia clitellata* from Australia and *Boeckella poopensis* of South America) are phylogenetically nested within paraphyletic groups of freshwater species, indicating that these species are most likely derived from common ancestors that lived in freshwaters (in contrast to Maly's (1996) proposal). On the other hand, the middle phases of Bayly's sequence remain somewhat

uncertain. It is not yet clear whether it is most common for marine taxa to move first into estuarine settings and then evolve the capacity to move inland, or whether the transition to freshwaters has occurred rapidly. However, single species can exhibit very broad ranges of salinity tolerance, as demonstrated by the two *Limnocalanus* species. Thus, prolonged estuarine phases do not appear to be physiologically essential for inland invasion in the centropagids. Addition of the remaining centropagid genera to the sequence dataset, and greater taxon sampling of marine species, will allow more detailed investigation into the history of habitat shifts.

In the most ancient continental invasion in the Centropagidae, it is clear that many subsequent shifts occurred among different types of inland waterbodies. One such move was from freshwaters to saline lakes, as observed independently in South America and Australia, but shifts between pond and lake environments were more common. Transitions between lotic and lentic environments are also observed; riverine and floodplain wetlands species (*B. bergi* and *B. fluvialis*) are found in both South America and Australia. These shifts may have played an important role in speciation. In the case of the *Boeckella* of Argentina, comparison of our phylogeny with the biogeographic track analysis of Menu-Marque et al. (2000) reveals some interesting patterns. While some sister clades differing in habitats are allopatric, indicating that the habitat shift could have occurred after allopatric divergence, other pairs live in regional sympatry, a pattern suggestive of speciation via habitat shift. For example, *B. bergi* inhabits rivers while its relatives *B. schwabei* and *B. gracilis*, whose distributions overlap with it, occupy ponds and lakes; *B. poopoensis* lives in saline waters while its sister species *B. meteoris* lives in freshwater lakes and ponds within the same region; and *B. diamantina* lives in large lakes while members of its sister clade that live in the same region occupy ephemeral habitats (see Adamowicz et al., 2007 for COI phylogeny including this species). Lake-pond shifts have also been suggested to play a role in genetic divergence and speciation of other zooplanktonic microcrustaceans such as *Daphnia* and *Bosmina* (e.g. De Melo and Hebert, 1994; Taylor et al., 1996; Pfrender et al., 2000).

4.3. Continental invasion and patterns of diversification

A previously noted pattern of diversification is that the timing of colonization is related to the extant diversity of freshwater copepod taxa (Boxshall and Jaume, 2000). This observation is understandable, in that groups with a longer period of continental occupancy would have had more time to accumulate species. The present study provides a phylogenetic framework for one of the dominant continental copepod families and will contribute to future quantifications of the strength of this pattern, once phylogenies of other groups become available. Moreover, detailed phylogenetic information will allow questions to be asked about the shape of species accumulation curves, and whether diversification rates change over time since colonization.

It is also possible that diversification rates differ between marine and continental lineages. For example, within the calanoid superfamily Diaptomoidea, primarily freshwater families are relatively diverse compared to the mainly marine ones. The freshwater family Diaptomidae contains about 515 species (Boxshall and Jaume, 2000), and Centropagidae contains 110 species, 60 of these living inland. By contrast, eight of the nine marine families in this superfamily contain fewer species. While a phylogenetic perspective is necessary for studying diversification rates, as taxa of the same rank can be of different evolutionary ages, these values suggest that it is worth investigating whether diversification rates increase upon continental invasion. Other suggestive evidence is found in the rapid pace of diversification of some recently invading clades, such as the “post-glacial” continental radiations of *Euryte-*

mora (with 27 species) and *Salmincola* (38 species) (see Boxshall and Jaume, 2000). However, the marine sister groups of inland copepod lineages are not generally well known at present. Future phylogenetic tests could include comparisons of diversity in sister clades inhabiting different environments and whole-tree analyses for detecting nodes exhibiting significant shifts in diversification rate (Chan and Moore, 2002, 2005).

4.4. Phylogeny, taxonomy, and morphology

For the most part, the phylogenetic evidence supports the current taxonomic system. The main exception is the case of *Parabroteas*, which was nested within a paraphyletic *Calamoecia*. Whether splitting or lumping is therefore in order is a subject for further consideration. Most morphospecies were recovered as monophyletic with little intraspecific variation, generally up to a maximum of 3.5% (K2P) in 16S and 0.4% in 28S. There was almost no overlap with the ranges of congeneric inter-species divergences, 4.0–18.6% in 16S and 0.2–5.6% in 28S. As such, these results involving one mitochondrial and one nuclear ribosomal rRNA gene parallel patterns of genetic variation in the protein-coding mitochondrial COI gene found among Argentine members of the genera *Boeckella* and *Parabroteas* (Adamowicz et al., 2007). Levels of divergence were typically higher in the faster-evolving COI gene when compared with the same species. Intraspecific divergences were observed up to a maximum of 4.1% and 6.3% within two morphospecies (more typically <2.5% within species), and congeneric inter-species divergences were 10.9–25.0%. Importantly, discontinuities between levels of intraspecific and interspecific divergence are observed in all three genes. Thus, a barcoding approach to species identification focusing on COI (Hebert et al., 2003), shown to be highly effective amongst a variety of crustaceans (Costa et al., 2007), in fact reflects an underlying pattern of genetic divergence at a variety of markers.

Two species in this study, *Boeckella michaelsoni* and *Calamoecia tasmanica subattenuata*, each contained two genetic lineages displaying a level of divergence typical of the interspecific patterns described above. These results imply the presence of putative cryptic species and call for further investigation involving other evidence, such as allozymes, ecology, and morphology. Bayly (1979) noted two morphological forms of *C. tasmanica* in Western Australia, one being *C. t. subattenuata* and the other not formally described, and suggested that at least three separate species, including *C. tasmanica* s.s., may be subsumed under the name *C. tasmanica* s.l. The specimens from Lake Joondalup displayed all the morphological characteristics of *C. t. subattenuata*. Similarly, those from Kulicup Swamp were more similar to *C. t. subattenuata* than to Bayly's (1979) undescribed morphotype, with one exception: they lacked the second spine on the distal exopod segment of the male fifth leg that is typical of *C. t. subattenuata*. Thus, the genetic differentiation between these samples was surprising. Additional samples and morphological work are required to determine the total extent of diversity within this species complex. The levels of genetic divergence between species pairs *B. gracilipes/B. titicacae* and *B. gracilis/B. schwabei*, which are sometimes considered synonyms, are consistent with their status as separate species. Although these pairs exhibit genetic distances that are towards the lower end of the interspecific range, the combined evidence currently supports species status, as these species were originally described based upon morphological differences.

Bayly's (1992c) synonymization of the former genus *Pseudoboeckella* with *Boeckella* is also supported, as separating them would result in a paraphyletic *Boeckella* when including the species from Australasia. Members of the former *Pseudoboeckella* (represented by *B. poppei*, *B. antiqua*, and *B. brasiliensis* here) formed a monophyletic group in the 16S phylogeny, but in a COI analysis

including more Argentine species, phylogenetic interspersation was observed amongst members of these former groupings (Adamowicz et al., 2007). The nested phylogenetic position of *Pseudoboeckella* within the (former) *Boeckella* as a whole is noteworthy, considering that it is the *Boeckella* species that were thought to have more unique and derived morphology (Brehm, 1956; Ringuelet, 1958). The molecular results caution against making assumptions about phylogeny solely on the basis of certain taxa exhibiting “reduced” character states, such as lower numbers of appendage segments and less setation (see Adamowicz and Purvis, 2006; Adamowicz and Sacherová, 2006; Adamowicz et al., 2007). A complex pattern of gain and loss of certain morphological traits is suggested for this group (Adamowicz et al., 2007), although more extensive character sampling is certainly needed for a full understanding of patterns of morphological evolution (Bayly and Boxshall, 2009).

5. Concluding remarks

Our molecular phylogenetic hypothesis for the broadly-distributed copepod family Centropagidae has answered several previous questions regarding its history of habitat transitions. Building upon this foundation by adding species, and investigating other groups, will enable future statistical tests of some key hypotheses about diversification, which we were only able to explore qualitatively here. For example, although the ocean is much larger in area and volume, the patchy nature and high diversity of inland water types may contribute to higher speciation (and extinction) rates. Rigorous tests of this idea will be fascinating in helping us to understand diversification in time and space. Biodiversity in the freshwater realm originates via a dynamic mixture of invasion from the sea and *in situ* speciation, whose relative contributions are beginning to be unraveled by combined molecular and biogeographic approaches.

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