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Molecular systematics and patterns of morphological evolution in the Centropagidae (Copepoda: Calanoida) of Argentina

SARAH J. ADAMOWICZ[1]*, SILVINA MENU-MARQUE[2], PAUL D. N. HEBERT[3] and ANDY PURVIS[1]

[1]*Division of Biology, Imperial College London, Silwood Park Campus, Ascot, SL5 7PY, UK*
[2]*Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 4to. Piso, C1428 EHA, Buenos Aires, Argentina*
[3]*Biodiversity Institute of Ontario, Department of Integrative Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada*

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Recent studies have shown the value of complementing standard taxonomy with genetic analyses to reveal cryptic diversity and to aid in the understanding of patterns of evolution. We surveyed variation in the COI mitochondrial gene in members of the three genera of centropagid copepods from the inland waters in Argentina. In general, we found a close association between molecular and morphological systematics in this group. Similar to findings for marine calanoids, genetic distances within *Boeckella* species were modest (< 4%), while distances among morphospecies were much larger (> 11%). *Parabroteas* is currently monotypic, although we detected cryptic genetic diversity, with two lineages showing 5.5% divergence. In contrast, *Karukinka* was not a valid genus, apparently representing an interesting and atavistic offshoot of *B. poppei*, a result reinforcing the value of considering both morphological and molecular evidence. Moreover, we used combined genetic and morphological information, analysed with maximum likelihood methods, to evaluate the common assumption that evolution tends to proceed via the loss of structures in crustaceans. Although analysis of other taxa and character types is required to evaluate fully the reduction hypothesis, our results suggest that structures may be gained readily as well as lost. *Biological Journal of the Linnean Society*, 2007, **90**, 279–292.

ADDITIONAL KEYWORDS: atavism – Bayesian analysis – character evolution – Crustacea – limb evolution – mitochondrial DNA – oligomerization – phylogeny – South America – zooplankton.

INTRODUCTION

Molecular analyses provide great promise for illuminating species boundaries in nature. While genetic studies have even revealed a few candidate cryptic species in well-known vertebrate groups (Hebert *et al*., 2004a), the potential for detecting new species is much greater in invertebrates (e.g. Gómez *et al*., 2002; Hebert *et al*., 2004b). In many cases, the kinds of variation that separate invertebrate species – such as ecological traits, aspects of physiological tolerance, and chemical mating cues – are not readily accessible to human observers (Knowlton, 1993). Variability in the accessibility of relevant traits among taxonomic groups has resulted in vast differences in the proportion of their total diversity that is currently described. For example, ratios of total-to-described diversity range from nearly 1 : 1 in well-known lepidopteran groups (Hebert *et al*., 2003) to ~1.7 : 1 within biogeographical regions for branchiopod crustaceans (Adamowicz & Purvis, 2005) and ~9 : 1 in a group of rotifers (Gómez *et al*., 2002). Ratios are much higher for bacteria, being perhaps 100 : 1 to 1000 : 1 or even

*Corresponding author. Current address: Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, ON, N2L 3G1, Canada.
E-mail: sadamowi@scimail.uwaterloo.ca

higher (Oren, 2004). In addition to identifying likely new species, combining genetic, morphological, and ecological information can reveal new insights about speciation and niche segregation in invertebrates not previously recognized based on morphology alone (Ortells, Gómez & Serra, 2003; Hebert *et al*., 2004b).

Among 'hard-to-identify' microinvertebrates, copepod crustaceans living in the marine realm in particular have received much attention from molecular biologists (e.g. Braga *et al*., 1999; Bucklin *et al*., 2003; Goetze, 2003). As they are thought to be the most abundant metazoans in the world (Huys & Boxshall, 1991), and are a key component of marine food webs, understanding their diversity and population dynamics is important for fisheries scientists and evolutionary biologists alike. Genetic tools are being developed for distinguishing species of marine copepods (Bucklin *et al*., 1999; Hill, Allen & Bucklin, 2001) and for investigating cryptic diversity (Goetze, 2003). In some calanoid families, there is a fairly close match between molecular and morphological species, even when geographically distant populations are sampled, although shallow intraspecific lineages have been detected (Bucklin *et al*., 2003). In another family, with a greater geographical extent of sampling within morphospecies, the fit was not good and many deep lineages and candidate cryptic species were revealed (Goetze, 2003).

While there is a growing pool of work on marine copepods, there are few studies evaluating species boundaries in freshwater copepods available for comparison with the marine results. In this study, we began to address this discrepancy by investigating the molecular systematics of freshwater members of the calanoid family Centropagidae. While this group contains marine species (and lineages that have moved into brackish and freshwaters in the Holarctic), this is by far the dominant group of calanoids in the freshwaters of southern South America and Australasia, a distribution thought to reflect the Gondwanan origin of members of this clade (Bayly, 1992a; Bayly *et al*., 2003). We included as many members as possible of the three genera within this family that are found in Argentina: *Boeckella*, which contains 17 species in Argentina and ~40 worldwide (Bayly, 1992b; Menu-Marque & Zúñiga, 1994; Menu-Marque & Balseiro, 2000), *Parabroteas*, which is monotypic (Bayly, 1992a), and the recently described and monotypic *Karukinka* (Menu-Marque, 2002). This copepod group represents a good candidate for the combined use of morphological and molecular approaches, because of the particularly high levels of intraspecific variation in certain characters present in a number of species. For example, *B. poppei* and *B. poopoensis* exhibit substantial variation in the structure of the fifth limbs (Paggi, 1983; Bayly, 1992a, b; Bayly *et al*., 2003), while there is a large degree of body size variation within and among populations of several species (Harding, 1955; Bayly & Burton, 1993). Moreover, two distinct size classes of mature individuals are seen in both sexes in some populations of *B. meteoris* and in females in *B. gibbosa* (Bayly, 1992b; S. Menu-Marque, pers. observ.; H. Zagarese, pers. comm.). Thus, while cryptic genetic diversity is often detected in marine copepods, current morphological taxonomy in the Argentine freshwater centropagids suggests high levels of morphological variation within single species, a supposition we aimed to test.

Another advantage of combining molecular and morphological approaches is the opportunity to study patterns of evolution. An alleged trend thought to be important in the evolution of crustaceans (and many other animal groups; Dogiel, 1954) is one termed 'oligomerization'. This is the tendency for serially homologous structures (such as appendage segments) and types of armature (such as spines) to evolve primarily by fusion, loss, or reduction during the course of evolution (Dogiel, 1954; Huys & Boxshall, 1991; Monchenko & von Vaupel Klein, 1999). In the copepods, raw trait counts are often used to draw conclusions about phylogenetic relationships. Again, the Centropagidae is a promising group for studying this purported trend. For example, in the genus *Boeckella*, the oligomerization principle has been used to suggest that the species *B. antiqua* may be the sister taxon to all other species in the genus, because of its high counts of appendage segments, spines, and setae (Menu-Marque & Balseiro, 2000). Similarly, *K. fueguina* was proposed to resemble the ancestor of *Boeckella* (and to be the sister group to that entire genus) because of its possession of a large number of 'primitive' swimming setae on the fifth limb, in contrast to the more bare and fewer-segmented limbs found in *Boeckella* species (which are instead used for grasping mates and spermatophore transfer) (Menu-Marque, 2002). Molecular phylogenetic information provides an important and independent source of data that can be used for testing such morphological hypotheses.

In this study, we evaluated the fit between molecular data and morphological taxonomy for most Argentine members of the family Centropagidae. Moreoever, we present here the first molecular phylogenetic hypothesis for this group. We used this phylogeny, in combination with a matrix of morphological characters, to investigate the question of whether morphological evolution has tended to proceed by the loss of structures in this group.

METHODS

Specimen collection

Specimens of *Boeckella* were collected from lakes and ponds throughout Argentina (Table 1) and preserved

Table 1. Collection localities of 12 species of *Boeckella*, *Karukinka fueguina*, and *Parabroteas sarsi* from Argentina

Species (population code)	Collection locality	Collector	Isolate no(s): COI GenBank accession no.
B. antiqua	Laguna Los Juncos, Río Negro	EB & HZ	1: DQ356541 2: DQ356542*
B. bergi	Pond by dirt road near Perito Moreno glacier, Santa Cruz	MCM	1: DQ356543*
B. brasiliensis (A)	Lago Toro, Chubut	HZ	A1: DQ356544*
B. brasiliensis (B)	Laguna San Luis, Tierra del Fuego	SMM	B1: DQ356545 B2: DQ356546
B. brevicaudata	Ditch, 6 km W of Tierra Mayor River, Rt. J, Tierra del Fuego	MCM	1: DQ356547*
B. diamantina	Laguna Barrosa, Mendoza	VF & AO	1: DQ356548 2: DQ356549* 3: DQ356550
B. gibbosa	Marsh at the head of Yaucha River, Mendoza	VF & AO	1: DQ356551* 2: DQ356552 3: DQ356553
B. gracilipes	Pond near km 2246 of Rt. 3, north of San Julián, Santa Cruz	MCM	1: DQ356554*
B. gracilis	Laguna Salada Grande, Buenos Aires	SMM	1: DQ356555* 2: DQ356556
B. meteoris	Pond near km 2193 of Rt. 3, Santa Cruz	SMM	1, 2: DQ356557 3, 7, 8: DQ356558* 4: DQ356559 5: DQ356560 6: DQ356561
B. michaelseni	Puddle near km 2923 of Rt. 3, Tierra del Fuego	MCM	1, 2: DQ356562 3: DQ356563 4: DQ356564*
B. poopoensis (A)	Laguna los Horcones, Buenos Aires	SMM	A1: DQ356565* A2: DQ356566
B. poopoensis (B)	Artificial pond in oil field close to Cañadón Grande, Chubut	AG	B1: DQ356567
B. poppei (A)	Ditch along provincial Rt. #1 to Cabo Vírgenes at km 85, Santa Cruz	MCM	A1: DQ356568*
B. poppei (B)	Same pond as *Karukinka* (see below)	SMM	B1: DQ356569 B2,B3: DQ356570
Karukinka fueguina (proposed synonym of B. poppei)	Type locality (small, temporary, roadside pool lying at the bottom of a gravel pit, Tierra del Fuego, at 54°09′S, 67°15′W)	SMM	1: DQ356576*
Parabroteas sarsi (A)	Pond near Sarmiento, Chubut (7 km north of Petrified forest reserve)	SJA	A1: DQ356571*
P. sarsi (B)	Ditch along Rt. #1, 82 km east of junction with Rt. #3, near Cabo Vírgenes, Santa Cruz	SJA	B1: DQ356572
P. sarsi (C)	Pond near Estancia María Behety, Rt. C, Tierra del Fuego	SJA	C1: DQ356573
P. sarsi (D)	Road-side ditch off Rt. 3, just south of Río Chico, Santa Cruz	SJA	D1: DQ356574*
P. sarsi (E)	Pond along Rt. # 40, 59 km north of Tres Lagos, Santa Cruz	SJA	E1: DQ356575

Population codes are provided when more than one population was available for a species.
Collections were made by Esteban Balseiro (EB), Verónica Fuentes (VF), Alfonso Giudici (AG), María Cristina Marinone (MCM), Alejandro Olariaga (AO), Horacio Zagarese (HZ), and the authors, while all determinations were made by one of the authors (SMM).
*Isolates used for Bayesian analysis.

in 96% ethanol. Species determinations were made by S.M.-M. using Bayly's (1992ab) keys and recent descriptions for *B. antiqua* Menu-Marque & Balseiro 2000, *B. diamantina* Menu-Marque & Zúñiga 1994, and *B. gibbosa* (Brehm, 1935), redescribed by Locascio de Mitrovich & Menu-Marque (1997). Twelve species of *Boeckella* were available, nine from a single locality each and three from two locations (Table 1). Part of the paratype specimen of *K. fueguina* Menu-Marque, 2002, preserved in ethanol, was used for DNA extraction. *P. sarsi* (Daday) was sampled from five localities.

Mitochondrial DNA analysis

DNA sequencing

Prior to DNA extraction, individuals were soaked in double-distilled water for a minimum of 1 h. DNA extractions of single individuals, with a total volume of 50 μL, were then prepared for one to four individuals per population, depending on the availability of individuals, using the proteinase-K method (Schwenk *et al*., 1998). Additional attention was paid to the population of *B. meteoris*, as there were two size classes of mature individuals. The 'large' group contained mature males and females, which were 1.5–1.6 times longer than were males and females in the 'small' group (S.M.-M., unpubl. data). Four individuals of each size class were sequenced.

A 710-base pair (bp) fragment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene was amplified from each individual via the polymerase chain reaction (PCR) (Saiki, Gelfand & Stoffel, 1988) using the primers LCOI490 and HCO2918 (Folmer *et al*., 1994). The PCR and sequencing protocols followed those of Adamowicz, Hebert & Marinone (2004). Sequence electropherograms were inspected and aligned using the SeqApp 1.9 sequence editor (Gilbert, 1992), with the aid of amino acid translation, resulting in a final alignment of 636 bp. All unique sequences have been deposited in GenBank under accession numbers DQ356541–DQ356576.

Sequence statistics and phenetic analysis

Nucleotide frequencies and transition/transversion ratios were calculated using MEGA 2.1 (Kumar *et al*., 2001). Since differences in nucleotide composition among sequences can adversely affect phylogenetic inference, the chi-square homogeneity test was used to test for significant departures from uniformity in nucleotide composition.

Divergence between all pairs of unique sequences was estimated using Kimura's (1980) two-parameter model (K2P) and pairwise deletion of the few missing nucleotides. Sequence variation was characterized at the intrapopulation, intraspecific, and interspecific levels. The distance matrix was then used to construct a phenogram in MEGA by the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987). *P. sarsi* was used to root the tree, since prior analysis of nuclear ribosomal sequences (28S) from several centropagid genera confirmed that this species was an appropriate choice, being closely related to, but not part of, a *Boeckella* plus *Karukinka* clade (S.J.A., unpubl. data). Bootstrap values were calculated using 1000 pseudoreplicates.

Bayesian analysis

Phylogenetic relationships were also reconstructed using Bayesian analysis in the program MrBAYES version 3.0b4 (Huelsenbeck & Ronquist, 2001). The dataset was first reduced to include a single member of each *Boeckella* species, plus *K. fueguina*, and two *P. sarsi* sequences for outgroups (Table 1). Sequences for each species were selected based on the criterion of minimizing the number of undetermined nucleotides (Ns) in this analysis, resulting in only three Ns among the 15 sequences.

Modeltest version 3.06 (Posada & Crandall, 1998) was used to select the best model of nucleotide substitution for the data. The Bayesian analysis was started with a random tree, and four Markov chains were run simultaneously. The Markov chain Monte Carlo (MCMC) analysis was run for 130 000 generations, with trees sampled every ten generations. The first 3000 trees sampled were discarded as the 'burn-in' phase. The remaining 10 000 trees sampled were used to a construct a 50% majority-rule consensus tree, with compatible nodes having lower support also being included. Nodal support values (posterior probabilities) were calculated as the percentage of the 10 000 sampled trees containing the node. Three independent runs of the MCMC analysis were performed to confirm that separate analyses converged on the same result.

Analysis of character evolution

Morphological data

Fourteen characters (Table 2), mostly involving the fifth thoracic limbs, were selected for analysis because they are known to vary in this group. Nine of these involved spine numbers on different parts of the limbs and five related to the number of segments in the exopods and endopods of the limbs (Fig. 1). The spine and segment characters were analysed separately. Among the 13 ingroup taxa and one outgroup species, there were missing data in just four cells (Table 3). Additionally, there were nine cases of intraspecific polymorphism involving two characters.

Table 2. List of morphological characters scored in this analysis

Character number	Character
Characters involving spine number	
i	# of spines or setae on the Ri of the right male P5
ii	Presence/absence (1/0) of spines or setae on the Ri of the left male P5
iii	Presence/absence of small spine on the inner side of the left claw of the male P5
iv	Presence/absence of spines on the inner side of the female P5 Re: 1 = at least some spines on the inner side (besides the three distal ones); 0 = no spines on the inner side (only the three distal ones present)
v	Ornamentation of the Ri of the male P5: 2 = with swimming setae, 1 = with spines at least on the right Ri, 0 = none
vi	Presence/absence of hairs on the inner border of the left Re1 of the male P5
vii	Presence/absence of a spine on the inner side of the right hook of the male P5
viii	Presence/absence of a spine at the end of the hook (Re3) on the right male P5
ix	Presence/absence of a spine at the end of the Re3 on the left male P5
Characters involving segment number	
i	Suture between B2 and endopods of the male P5 either clearly visible (1) or nearly/completely fused (0)
ii	Maximum number of segments of the Ri of the right male P5 (2 or 3), or unsegmented (1)
iii	Maximum number of segments of the Ri of the left male P5 limb (2 or 3) or unsegmented (1)
iv	Hook (Re3) on the right male P5 limb segmented (1) or unsegmented (0)
v	Hook (Re3) on the left male P5 segmented (2), unsegmented (1), or reduced/absent (0)

P5, fifth leg; Re, outer ramus (exopod); Ri, inner ramus (endopod).

Character evolution under maximum likelihood

Maximum likelihood (ML) methods were used to analyse the character data. ML approaches have the advantage of incorporating branch-length information into the reconstruction and, importantly, provide a statistical framework for testing hypotheses about alternative evolutionary scenarios (Schluter *et al*., 1997; Mooers & Schluter, 1999; Pagel, 1999a, b).

ML analyses were conducted in Mesquite version 1.05 (Maddison & Maddison, 2004a) using the StochChar module version 1.05 (Maddison & Maddison, 2004b). As polymorphisms were not permitted by this program for ML analyses, species were conservatively assigned the higher character state observed (assuming that the lower states had resulted from loss, or oligomerization). Those taxa with a missing character state were trimmed from the phylogeny for the analysis of that character alone, but included for other characters for which they had data. Character states were rescored using consecutive numbers starting from zero, as required by the program for analysing discrete traits. Analyses were performed using both Bayesian and NJ phylogenies, with branch lengths ultrametricized prior to analysis, because we were more interested in branch lengths as a surrogate for time, than we were in analysing correlates of rates of molecular evolution. ML analyses were conducted omitting the highly unusual taxon *K. fueguina* (because of its large number of different morphological features and the extremely short branch leading to this taxon).

Three models of character evolution were applied to each character: an 'asymmetrical' model, in which separate forward and reverse transition rates were estimated; a 'one-rate' model, in which a single evolutionary rate was estimated (which would apply to both gains and losses); a 'loss-only' model, in which the forward rate was constrained to be 1^{-10} and a reverse rate was freely estimated. (Constraining the rate to be zero caused a program malfunction; results were virtually identical when rates of 10^{-9} or 10^{-8} were used.) The oligomerization hypothesis was tested by comparing the loss-only and asymmetrical models using a likelihood-ratio test (LRT) (Goldman, 1993; Pagel, 1994). Since the models being compared were nested, the LRT statistic was expected to follow a chi-square distribution. There was a single degree of freedom (i.e. a difference of one parameter between models) in the tests involving single characters. Groups of characters (spines and segments) were also evaluated using a pooled test, in which –ln likelihood scores were summed across characters for each model. In this case, the number of freely estimated parameters was equal to the total number of characters in the test. In addition, the hypothesis of asymmetry in forward and reverse rates was tested by comparing the one-rate and two-rate models using an LRT. Rejection of the one-rate model could indicate a general bias or heterogeneity in bias among characters. Two-tailed tests were performed in all cases.

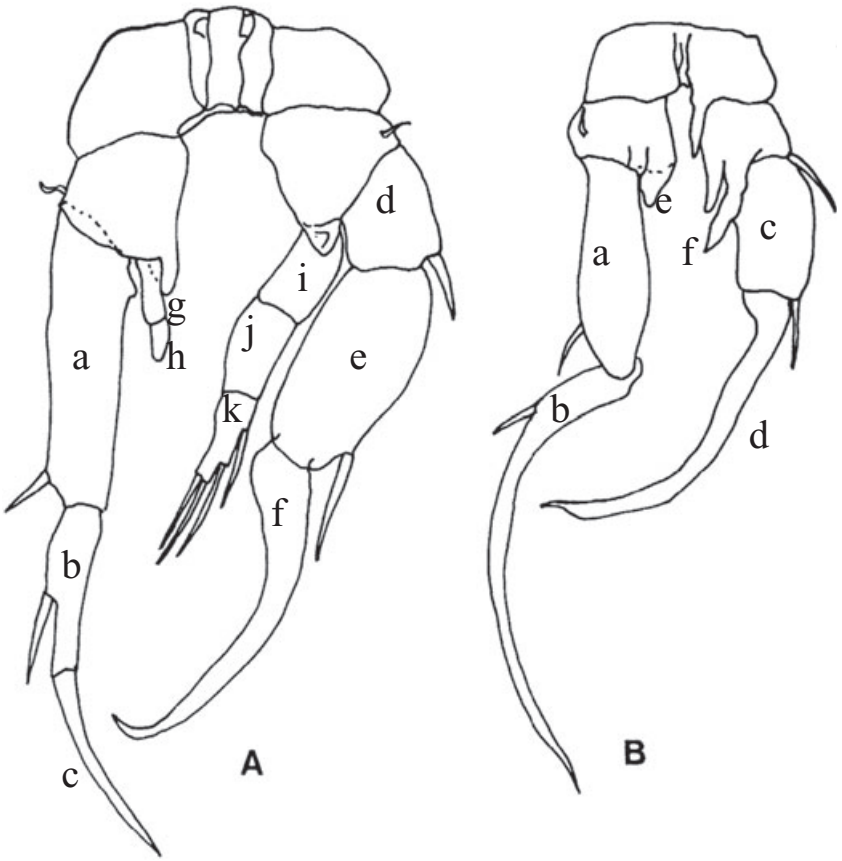

Figure 1. Diagrams of the fifth pair of legs of male copepods, shown from the caudal view. A, *Boeckella poppei* male, which displays the maximum number of endopod and exopod segments observed among the study taxa. The limb parts are labelled as follows: a–c, segments of the left exopod; d–f, segments of the right exopod; g,h, segments of the left endopod; i–k, segments of the right endopod. B, *B. bergi* male, which displays the most oligomerized state observed. The limb components are: a,b, segments of the left exopod; c,d, segments of the right exopod; e, reduced left endopod; f, reduced right endopod.

RESULTS

Sequence composition and diversity

There were 30 unique haplotypes among the 35 individuals sequenced from 12 *Boeckella* species (Table 1). Additionally, a single sequence from *K. fueguina* and five unique sequences from *P. sarsi* were obtained. The average nucleotide composition among the 36 sequences was: T, 37.5% (range, 35.2–39.5%); C, 15.2% (range, 13.1–17.5%); A, 27.3% (range, 26.1–30.0%); G, 20.0% (range, 19.2–22.0%). While all sequences were A–T biased, nucleotide composition did not differ significantly among them ($\chi^2 = 48.2$, d.f. = 105, $P > 0.999$). The average transition : transversion (Ts : T) ratio was 1.34. Transitional saturation (Ts : Tv = ~1) occurred at pairwise K2P distances of approximately 20% and greater.

Intrapopulation COI sequence divergence was generally very limited. Multiple individuals were sequenced from the same population for nine species of *Boeckella* (Table 1). Pairwise distances between individuals were 0.0–0.6% in eight of the nine cases. The remaining species, *B. meteoris*, exhibited a much higher level of divergence among individuals (mean, 2.4%; maximum, 4.1%). Although this population showed substantial size variation among adults, the divergent haplotypes were not associated with size class.

Intraspecific sequence variation among individuals from different populations of single *Boeckella* species also tended to be low. Mean interpopulation divergences ranged from 0.1 to 2.4% for the three species that were examined. *P. sarsi*, which was sampled from five geographically widespread sites, displayed greater intraspecific genetic diversity, with pairwise divergences varying from 0.3 to 6.3%. Average divergence between the most different lineage of *P. sarsi* (from collection site A) and a tightly clustered grouping (B1-E1) was 5.5%.

Table 3. Character data for *Parabroteas sarsi*, *Karukinka fueguina*, and 12 species of *Boeckella*. See Table 2 for a description of the characters and character states

	Spine characters									Segment characters				
Species	i	ii	iii	iv	v	vi	vii	viii	ix	i	ii	iii	iv	v
Parabroteas sarsi	6,7	1	?	1	2	1	1	0	0	1	3	3	1	0
Karukinka fueguina	6,10	1	1	?	2	1	1	1	1	1	2,3	3	1	2
Boeckella poppei	4	0	0	1	1	1	0	0	0	1	3	2	0	2
B. brasiliensis	4	0	1	?	1	?	0	0	0	1	3	2	0	1
B. antiqua	4	1	0	1	1	1	0	0	0	1	2,3	3	0	2
B. gibbosa	8	0	0	1	1	0	0	0	0	1	2,3	1	0	1
B. diamantina	4	0	0	0	1	0	0	0	0	1	2,3	1	0	2
B. bergi	0	0	0	0	0	0	0	0	0	0	1	1	0	1
B. gracilis	0	0	0	0	0	0	0	0	0	0	1	1	0	1
B. meteoris	0	0	0	0	0	0	0	0	0	1	3	2	1	2
B. gracilipes	0	0	0	0	0	0	0	0	0	1	2,3	1	1	2
B. poopoensis	0	0	0	0	0	1	0	0	0	1	2	2	0	2
B. brevicaudata	1,2	0	0	0	1	0	0	0	0	1	3	1	1	2
B. michaelseni	0	0	0	0	0	1	0	0	0	1	2,3	1	0	1

Interspecific divergence was substantially greater than that within species, and there was no overlap in the ranges of variation observed. Average K2P distances among species within the genus *Boeckella* varied from 10.9 to 25.0%. Divergence between the various *Boeckella* species and *P. sarsi* ranged from 16.8 to 25.8%. In contrast, the *K. fueguina* sequence showed only 1.0% divergence from three individuals of *B. poppei* collected from the same locality.

Phenetic analysis of all sequences

The NJ tree based on all unique COI haplotypes recovered clusters that corresponded to the recognized species of *Boeckella* (Fig. 2). Each of these clusters was supported by a bootstrap value of 100, and was separated by long branches from other species. Two species (*B. meteoris* and *P. sarsi*) also exhibited intraspecific clusters. The *K. fueguina* sequence clustered among the *B. poppei* isolates, being closest to those individuals collected from the same habitat. In general, bootstrap values at the deeper nodes in the NJ tree were low to moderate, ranging from 26 to 77%.

Bayesian phylogenetic analysis

Modeltest indicated that the TVM + I + G sequence evolution model provided a significantly better fit to the sequence data than did simpler models. Thus, the options selected for Bayesian analysis were the six-parameter substitution rate matrix (rather than one or two parameters), gamma parameter, and invariant sites parameter, with the specific values estimated by MrBAYES. The consensus tree from the Bayesian analysis provided a dichotomous phylogenetic hypothesis for the genus (Fig. 3). Most nodes were present in > 70% of the sampled trees, but three nodes had low (35%) or moderate (57%, 60%) posterior probability. In each of the three independent MCMC analyses, the same topology and similar nodal support resulted. Thus, only the results of the first analysis are presented here.

Most of the species formerly placed in the genus *Pseudoboeckella* formed a single, well-supported clade (with 99% support) (Fig. 3). The sole exception was *B. brevicaudata*, a species whose placement has always been problematic (Bayly, 1992b). *K. fueguina* was also a member of this group, showing a close affinity to *B. poppei*. Species formerly assigned to the genus *Boeckella*, along with *B. brevicaudata*, formed three clades and displayed a paraphyletic relationship to former members of *Pseudoboeckella*.

Analysis of character evolution

The ML results based on the Bayesian and NJ phylogenies were very similar, and thus only the former are reported here. The likelihood ratio tests rejected the strict oligomerization hypothesis, as indicated by the significantly worse fit of the loss-only model compared with the two-rates model (Table 4). This result was consistent across both spine and segment character groups. Considering individual characters, seven of 12 characters rejected the oligomerization hypothesis on their own, despite the small sample size of species.

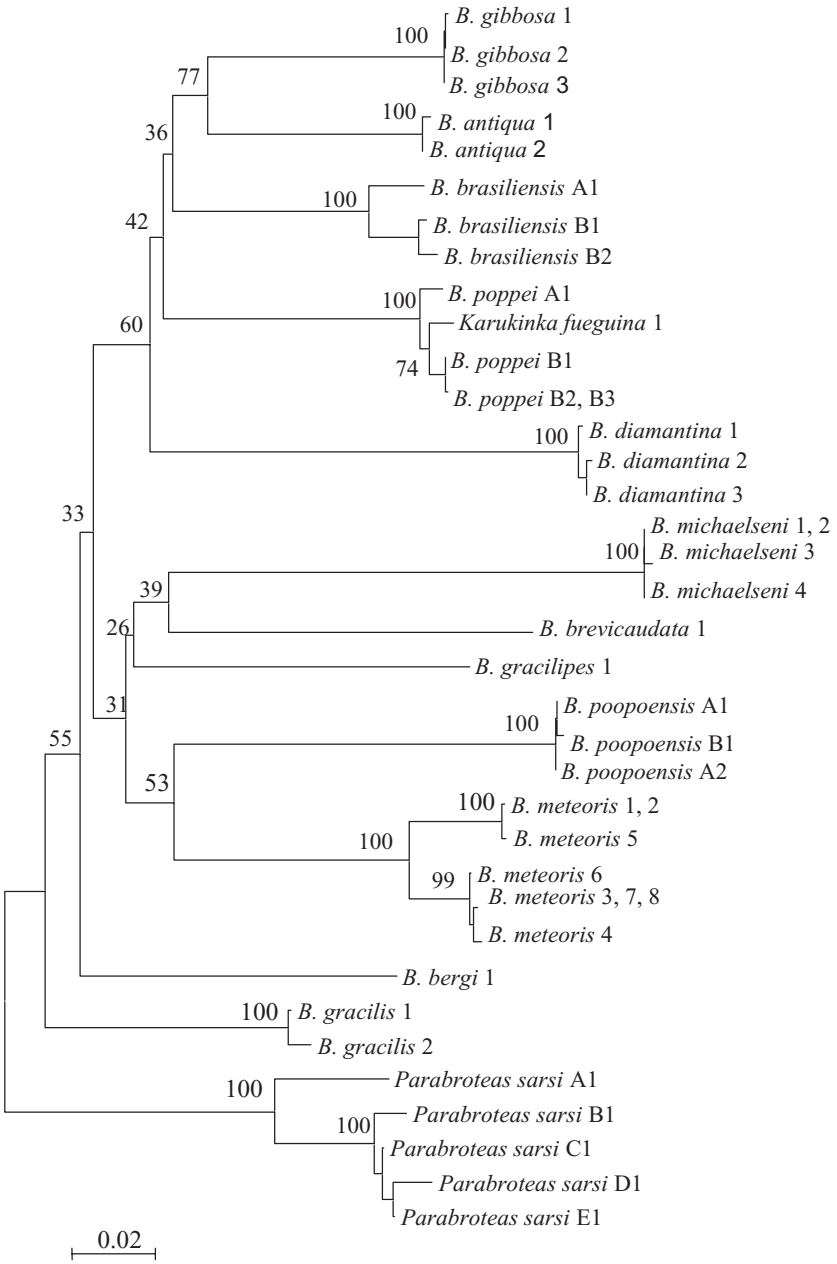

Figure 2. Neighbour-joining tree based on COI sequence variation among all haplotypes of 12 *Boeckella* species from Argentina, *Karukinka fueguina*, and *Parabroteas sarsi*. The analysis was performed in MEGA and rooted with *Parabroteas*. Codes for isolates and collection localities are listed in Table 1. Individuals 1–4 of *B. meteoris* were of small body size, while individuals 5–8 were large. Bootstrap values based on 1000 pseudoreplicates are provided for selected nodes. The scale bar indicates Kimura's (1980) two-parameter model genetic distance.

LRTs also indicated that the two-rate model was not a significantly better fit to the data than was the single-rate model either for the pooled spine characters or for the pooled segment characters. Among individual characters, none of the characters favoured the two-rate over the one-rate model, although two characters showed a marginally non-significant ($P < 0.10$) better fit for the two-rate model (Table 4). Although there was no statistical support for a bias towards either gains or losses, we noted that nine of 12 characters showed a reconstructed loss bias using the two-rate model. Moreover, the median bias across characters (i.e. the median ratio of gain rate : loss rate) was 0.38.

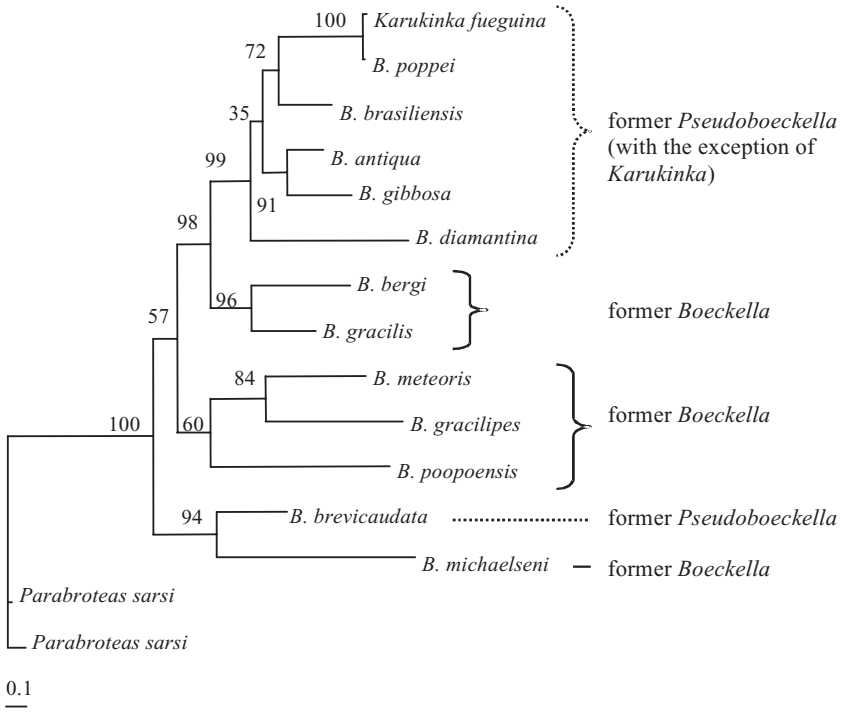

Figure 3. Majority-rule Bayesian tree for species of *Boeckella*, *Karukinka*, and *Parabroteas*. The membership of each species of *Boeckella* in the former genera *Boeckella* or *Pseudoboeckella* is indicated, according to Bayly's (1992b) list of morphological distinctions. The scale bar shows maximum likelihood distance. Numbers above nodes are the percentage of the 10 000 sampled trees containing that node.

DISCUSSION

Molecular vs. morphological systematics

We have presented the first molecular phylogenetic investigation of South American members of the calanoid family, Centropagidae. As for many other microinvertebrates, this proved to be a valuable tool for evaluating the fit between genetic groupings and species boundaries as recognized by morphological taxonomists. In general, for the genus *Boeckella* the molecular results strongly supported the existing taxonomic system, indicating that the kinds of morphological differences recognized by taxonomists reflect underlying genetic groupings.

We revealed tight genetic clustering within morphospecies, with intraspecific divergence of less than ~2.5% in most cases, but up to 4.1% in the COI gene for *B. meteoris*. Although in some *Boeckella* species we were able to sample individuals from only one habitat, and further sampling is required, this genetic clustering was observed even among the most morphologically variable species. The two distinct size categories of mature individuals within *B. meteoris* did not correspond to monophyletic mitochondrial groups, suggesting that these size groups are part of a single species. It would be interesting to confirm this result by looking at nuclear markers (e.g. allozymes), as hybridization cannot be ruled out by the mitochondrial results as a potential genetic explanation for the size variation. Ecological reasons for this size dimorphism are not known, although this could represent a phenotypically plastic response enhancing food–resource acquisition. Similarly, all *B. poppei* haplotypes, even those from different habitats, were very closely related, although this is the most morphologically variable species in the genus (Paggi, 1983; Bayly *et al*., 2003), and individuals sampled here showed variation in the male P5 limb. In addition to the clustering observed within species, we found that morphospecies were well separated genetically, with a minimum divergence of 10.9% observed between pairs of species in the genus. Finally, our results for *Boeckella* also agree with the conclusion of Bayly (1992b), based on his morphological studies, that species of the former genera *Boeckella* and *Pseudoboeckella* are correctly assigned to a single genus.

Similarly, molecular results for *P. sarsi* were largely in agreement with current taxonomy, in which this genus is monotypic. However, two distinct lineages were detected among the five populations sampled, suggesting the possibility of a cryptic species, although the genetic divergence (~5.5%) between

Table 4. Results of likelihood ratios tests (LRTs) comparing the likelihood (L) scores of three models of character evolution

	Two-rate model		Single-rate model			Loss-only model		
Character	Reconstructed bias (forward:reverse rate)	–ln L	–lnL	LRT statistic	Worse fit than two-rates?	– lnL	LRT statistic	Worse fit than two-rates?
Spine i	0.225	15.36	15.84	0.96	$P = 0.33$	19.06	7.40	$P = 0.0065$**
Spine ii	0.182	4.89	5.64	1.50	$P = 0.22$	9.66	9.54	$P = 0.0020$**
Spine iii	0.091	2.75	4.08	2.66	$P = 0.103$	4.75	4.00	$P = 0.0455$*
Spine iv	1.038	5.59	5.59	0.00	$P = 1.00$	7.60	4.02	$P = 0.0449$*
Spine v	0.347	9.07	9.21	0.28	$P = 0.60$	9.74	1.34	$P = 0.247$
Spine vi	0.714	7.46	7.62	0.32	$P = 0.58$	10.31	5.70	$P = 0.0169$*
Spine vii	0.064	2.10	2.50	0.80	$P = 0.37$	2.14	0.08	$P = 0.777$
Pooled (d.f. = 7)		47.21	50.49	6.56	$P = 0.476$	63.27	32.12	$P < 0.001$***
Segment i	7.065	4.09	4.15	0.12	$P = 0.73$	4.11	0.04	$P = 0.841$
Segment ii	0.00012	7.52	7.61	0.18	$P = 0.67$	7.52	0.00	$P = 1.00$
Segment iii	0.413	11.72	13.18	2.92	$P = 0.087$ ~	16.37	9.30	$P = 0.0022$**
Segment iv	0.750	6.42	6.44	0.04	$P = 0.84$	7.11	1.38	$P = 0.240$
Segment v	2.732	11.20	12.90	3.40	$P = 0.065$ ~	13.85	5.30	$P = 0.0213$*
Pooled (d.f. = 5)		40.95	44.30	6.70	$P = 0.244$	48.97	16.04	$P = 0.0067$**
Median bias:	0.38							

A significantly worse fit of the 'loss-only' (compared with the two-rate) model would result in the rejection of the hypothesis of unidirectionality. A worse fit of the one-rate model (compared with the two-rate) would provide evidence for a complex pattern of character evolution (either general bias in forward vs. reverse evolutionary rates or heterogeneity in bias among characters). Each test involving a single character had a single degree of freedom (d.f). Spine characters viii and ix have been omitted, as there was no remaining variation once the highly unusual taxon *Karukinka* was excluded.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

these lineages was only about half the minimum level observed between pairs of *Boeckella* species. Thus, these cryptic lineages within *P. sarsi* should probably be maintained as conspecific, until further genetic, morphological, or breeding evidence is investigated.

In contrast, the very shallow divergence found between *K. fueguina* and *B. poppei* suggests that the recently described taxon *K. fueguina* Menu-Marque 2002 does not constitute a new genus, and perhaps does not even constitute a valid species. This striking discordance between molecular and morphological results compels further consideration of the origins of this taxon. The fact that individuals of *B. poppei* cohabiting with *K. fueguina* were more closely related to that taxon (1.0% COI divergence) than they were to their conspecifics from other localities (1.3% divergence) suggests that *K. fueguina* is a recent derivative of the widespread and more genetically variable *B. poppei*. Moreover, since only two male individuals of *K. fueguina* are known (Menu-Marque, 2002), it seems probable that this taxon is not a reproductively isolated, self-sustaining population. Possible explanations for the origin of these unique individuals include hybridization (with *B. poppei* as the maternal parent), polyploidy, atavistic macromutation affecting limb development, or some combination of these. If more individuals are found, a combination of sequencing, allozyme analysis, and genome size determination (as in Adamowicz *et al*., 2002) should help to reveal their status and origin. Here, we propose synonymizing *K. fueguina* with *B. poppei*, but, in light of the great morphological differences between them, the *Karukinka* specimens should be considered as highly aberrant individuals, likely to be either mutants or hybrids.

Comparison of freshwater and marine calanoids

Levels of genetic divergence within and among *Boeckella* species were similar to those observed in marine calanoids. For example, Bucklin *et al*. (2003) found that genetic distances within species of Calanidae and Clausocalanidae ranged from 1 to 4%, while divergence among species was at least 9%, compared with a minimum COI distance of 10.9% among species of *Boeckella*. Similarly, Goetze (2003) found that the youngest pair of already described species of Eucalanidae showed 3% divergence in the 16S gene, and 13.5% in the COI gene. However, undescribed lineages were detected in that family that surpassed this level of variation and may be considered candidate cryptic species, requiring further attention.

The similar levels of genetic variation observed in marine and freshwater calanoids bodes well for the possibility of developing a broad DNA-based species identification system for the group. Generally, projects aiming to 'barcode' all species are gaining momentum (e.g. Janzen, 2004) as a high rate of success at species identification is demonstrated (e.g. Hebert *et al*., 2003, 2004a). Such identification tools have already been developed for particular groups of copepod species, such as North Atlantic *Calanus* species (Hill *et al*., 2001). The construction of broader sequence databases for the identification of copepods at any life stage is underway as part of the ZooGene project (see Bucklin *et al*., 2003), and we have shown here that this group of freshwater calanoids would fit well into this effort. Such an identification system will be particularly useful for groups such as copepods, for which dissection of minute body parts of adult stages by taxonomic experts is currently required for accurate species determination.

One apparent difference, however, between our freshwater centropagids and marine calanoids is that we did not detect any clear cases of cryptic species. It seems that the high levels of morphological variation in some characters observed within described *Boeckella* species are accommodated within the species boundaries suggested by mtDNA analysis. However, further geographical sampling is necessary to include all morphological forms of some highly variable species, such as *B. poppei*. Nevertheless, one might reasonably expect greater cryptic diversity in (nearly) globally distributed species, such as many marine copepods, compared with those from a specific region of one continent. Further regional sampling within *Boeckella* should further clarify species boundaries and the limits of intraspecific morphological variation in the group.

Morphological evolution by oligomerization

Insights from Boeckella

The hypothesis of strict oligomerization – that serial structures evolve solely by loss or fusion – was not supported by ML analysis for our set of morphological characters. This highly significant rejection was obtained despite our small sample of species, suggesting that evolution via oligomerization is not a good description of evolution for the traits that we examined in this group of species. Although some of the deeper nodes in our COI phylogeny were poorly supported, the oligomerization results appear to be robust for four main reasons. First, the results of character analysis were very similar when based upon phylogenetic hypotheses derived using different phylogenetic methods and assumptions (e.g. maximum parsimony, NJ, and different treatments of transitions and transversions). Second, conclusions were similar when maximum parsimony was used to map character state transitions under various forward and reverse weighting schemes. Third, the oligomerization hypothesis was rejected even more strongly when the highly unusual taxon *Karukinka* was included. Finally, the strongly supported and 'nested' phylogenetic positions of the supposedly primitive species *B. antiqua* and *K. fueguina* themselves support the conclusion that morphological evolution has not always proceeded as traditionally supposed in this group.

Thus, our results for *Boeckella* suggest that structures have been gained repeatedly, rather than being only lost, during the evolutionary history of the group, in contrast to some previous interpretations of morphological variation in these species (Brehm, 1956; Ringuelet, 1958; Menu-Marque & Balseiro, 2000; Menu-Marque, 2002; Bayly *et al*., 2003). However, it is not clear whether this finding for *Boeckella* represents a more general challenge to the paradigm of 'evolution by reduction'. Many of the characters included here for their variability within the genus are located on the fifth limb of the male and are involved with grasping females and transferring the spermatophore. Thus, they may be subjected to strong sexual selection and might not follow the pattern of evolution seen in other crustacean groups and in other types of characters. However, the observation that our results were in conflict with previous expectations suggests that evolution by loss should not be accepted a priori. In particular, conclusions about phylogenetic relationships should not be based upon this assumption. In addition, it would be useful to re-evaluate assumptions about the directionality of ecological shifts (e.g. Smirnov, 1969) based on the oligomerization hypothesis.

Although our data led us to reject the hypothesis of strict unidirectionality, the true mode of evolution in these characters is not clear. Our ML analyses did not find a significant difference between the one-rate and two-rate models, so there is no statistical evidence that evolution favours losses over gains. However, this lack of significance may have resulted from low power, because of our small sample size. The median bias across characters was ~0.4, indicating about 0.4 gains for every loss. Thus, it may be that oligomerization in centropagid limb characters is manifested as a tendency towards loss, rather than as a hard-and-fast rule. Fine-tuning our understanding of patterns of character evolution in copepods and other crustaceans will require both larger sample sizes of characters and taxa and investigation at different taxonomic levels. Tests involving a broad variety of characters and taxa have indicated similar patterns in other crustacean groups, at least when looking at patterns of morphological variation at low taxonomic levels (Adamowicz & Purvis, 2006).

Insights from other studies

Phylogenetic studies of other crustaceans provide useful material for comparison, with additional examples contradicting the oligomerization principle found among the fairy shrimp (Class Branchiopoda; Order Anostraca). Weekers *et al*. 2002 point out that the species *Polyartemiella hazeni* and *Polyartemia forcipata* were formerly thought to be among the most primitive anostracans because of their possession of 17 and 19 thoracic segments and sets of trunk limbs, respectively, compared with the normal 11 found in the order. However, their molecular results strongly support the conclusion that the six and eight extra segments are derived traits. On the other hand, Remigio, Hebert & Savage (2001) provide evidence that at least two independent cases of segment loss (from 11 to ten) have occurred in the genus *Parartemia*, in agreement with the reduction rule.

The results of a recent molecular phylogenetic study of the branchiopod crustacean family Chydoridae also generally appear to conform to certain traditional expectations (Sacherová & Hebert, 2003). Their three-gene results produced a phylogeny for the four subfamilies that is concordant with previous hypotheses based on assessments of 'primitive' vs. 'derived' character states (Fryer, 1968; Smirnov, 1971). However, examination of the recent molecular phylogeny against a morphological character matrix (Smirnov, 1969) suggests that patterns of morphological evolution in this group may be more complex than those suggested by the subfamilial arrangement. Detailed character analysis of the chydorids at lower taxonomic levels using the phylogenies of Sacherová & Hebert (2003) suggests that many exceptions to the principle of reduction occur in this group as well (Adamowicz & Sacherová, 2006).

Outside of the Crustacea, based on analyses of a large morphological dataset, Brooks & McLennan (1993) argued that in the parasitic flatworms, a group in which evolution by trait loss had been presumed to be the norm, evolution does not proceed mainly through reduction. Indeed, only about 11% of their reconstructed character-state transformations were losses of structures. While it would be desirable to include molecular phylogenetic information in future studies of morphological evolution in parasitic flatworms, it appears that the challenge to the reduction paradigm is not confined to the crustaceans.

The studies mentioned above indicate that there are some cases in which the oligomerization hypothesis holds, but other cases in which it does not. Thus, even now, there is ample evidence indicating that oligomerization is not a hard-and-fast rule, but that it may now be considered a potential trend towards loss. Only additional work using molecular phylogenies on more taxa and more characters will reveal the true pattern of morphological evolution. In addition to phylogenetic work, important evidence may be obtained through an enhanced understanding of the ontogeny of morphological features. For example, differences in the timing of development of segments and sutures may reveal hidden differences between species having the same numbers of structures (Ferrari, 1988; Boxshall & Huys, 1998), providing insights into homologies and the mechanisms of character evolution.

Concluding remarks

The centropagid calanoids of Argentina show a close match between species boundaries determined by morphological taxonomists and those revealed by mtDNA analysis, suggesting that DNA-based identification systems could be developed for this group. However, our results also highlight the value of combining morphological and molecular information, as the interesting case of apparent atavism in *Karukinka* would have been missed if only one type of information were used. Moreover, to our knowledge, this is the first study to use molecular data to investigate the patterning of morphological transitions in a closely allied group of copepod species. Our data strongly reject the unidirectionality model. Although this result applies to one small taxonomic group and a particular assemblage of traits, character gains have been detected in other studies as well, and we suggest that these findings necessitate a re-evaluation of the general rule of oligomerization. These findings also reinforce the value of dialogue between the realms of morphology and molecules in studying evolutionary patterns. We expect that further integration of the fields of comparative morphology, molecular phylogenetics, and developmental biology will reveal much about the mechanisms of evolutionary diversification.

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