

Molecular evidence suggests an ancient radiation for the fairy shrimp genus *Streptocephalus* (Branchiopoda: Anostraca)

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Phylogenetic relationships among assumed Gondwanan aquatic inland invertebrate fauna are generally largely neglected, and biogeographical hypotheses for these organisms are generally inferred from historic (palaeogeographical) and contemporary distribution patterns. The distribution of the monogeneric thermophilic freshwater fairy shrimp family Streptocephalidae (*Streptocephalus*) provides a particularly useful framework to test the three contrasting biogeographical scenarios proposed for the evolution of this group: (1) the genus evolved in Laurasia and subsequently dispersed into Africa and North America; (2) the genus evolved and dispersed out of Africa and (3) the current distribution of the genus is the result of vicariance following the fragmentation of Gondwana. In the present study, the phylogenetic relationships of species in this genus are examined with the use of two mitochondrial genes (12S rRNA and COI mtDNA), while the phylogenetic relationships among the North American species and selected African taxa was investigated using the nuclear fragment (5.8S-ITS-1-18S). Phylogenetic results indicate that *Streptocephalus* probably evolved in Gondwana and that the current distribution patterns are a consequence of a combination of vicariance and limited dispersal. The implications for the evolution of continental freshwater crustaceans are discussed. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 82, 313–327.

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INTRODUCTION

The Anostraca, commonly known as fairy or brine shrimp, are a basal crustacean lineage with a fossil record that extends to the lower Cretaceous – 141 Mya (Fryer, 1987). A recent molecular study by Gaunt & Miles (2002) dates the divergence of this order from insects to between 434 and 421 Mya during the Silurian–Ordovician boundary, corroborating its antiquity and phylogenetic distinctiveness. Within the Anostraca, no large-scale modern cladistic analysis has been undertaken on any of the widely distributed genera in an attempt to examine the possible factors that have sculpted contemporary biogeographical patterns in this group of largely freshwater organisms. Such research is central in understanding the biodi-

versity and historical biogeography of freshwater invertebrate groups. While a Gondwanan or Pangaeian genesis has been advocated for a number of freshwater crustaceans, limited phylogenetic analysis has been undertaken on any of these faunas on a global scale (with the exception of research on freshwater crayfishes – Crandall, Harris & Fetzner, 2000) to determine the phylogenetic relationships within such groups (Schram, 1974; Poore & Humphreys, 1998). In this respect, the monotypic species-rich inland fairy shrimp *Streptocephalus* (Streptocephalidae) provides the ideal group to examine historic and contemporary factors that have sculpted the biogeography of freshwater faunal assemblages. Globally, this thermophilic genus consists of roughly 60 species and is present in all tropical and warm temperate areas of the Nearctic and Palearctic, excluding only South America (Belk & Brtek, 1995).

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Three contrasting hypotheses currently prevail on the evolution of this genus. Marked species diversity on the African continent led Wiman (1979) and Bănărescu (1990) to hypothesize that *Streptocephalus* possibly evolved in Africa and subsequently dispersed to Eurasia, Australia and recently to North America. This hypothesis predicts that species from each of these continental areas should form well-supported monophyletic units, with the Eurasian, Australian and North American species being more closely related, assuming a single out of Africa dispersal. Bănărescu (1990) is further of the opinion that, because *Streptocephalus* is the most thermophilic genus, the African continent, with its large heterogeneity in climatic regimes, and general absence of large ice covered areas, provided the most potential for speciation as opposed to the colder and more recently colonized North America. The dispersal hypothesis is partially supported by the desiccation-tolerant cysts that these animals produce, allowing passive dispersal and survival under extreme environmental conditions (Proctor, 1964; Saunders, Belk & Dufford, 1993; Skagen & Knopf, 1994; Bohonak & Whiteman, 1999; Wissinger *et al.*, 1999).

In contrast, Belk (1984) [ignoring the original description of a single species from Australia described by Sars (1896) and material re-collected later by Linder (1941)] proposed that the modern North American and African *Streptocephalus* species were derived from a common Laurasian stock and moved into Africa following the break up of Gondwana. This hypothesis was partially supported by the presumed absence of streptocephalids in South America and Australia. More recently, however, the presence of *Streptocephalus* has been confirmed in Australia with the description of a new species from Northern Queensland (Herbert & Timms, 2000), casting doubt on Belk's (1984) hypothesis.

Both Maeda-Martinez *et al.* (1995a) and Hamer *et al.* (1994a, b) defined nine morphological groups for the global streptocephalid fauna and suggested that each of these groups is probably monophyletic. Hamer *et al.* (1994a) admit that the designation of species into morphological groups was tentative and emphasized the need for a molecular phylogenetic study to corroborate or refute these findings. A cladistic analysis of morphological characters by Maeda-Martinez *et al.* (1995b) for all of the North American streptocephalids and selected species from Africa, Europe and India suggested that species from North America represent relictual forms of ancestral groups fragmented by continental drift, with some species possessing 'clear affinities with taxa on other continents'. These authors are further of the opinion that the genus probably had a Laurasian origin (early Cretaceous, 105 Mya) and dispersed through Africa,

Madagascar, India, Sri-Lanka, Indonesia and North America following the breakup of Gondwana (late Cretaceous, 60–70 Mya). Alternatively, Maeda-Martinez *et al.* (1995b) suggest that *Streptocephalus* had a Paangaean distribution and later became extinct or had not been discovered in South America or Australia.

Systematically, most of the subgeneric divisions in the Streptocephalidae have been unstable. Verill (1869) placed *Streptocephalus cafer* and *Streptocephalus similis* in the subgenus *Heterobranchipus*. Daday (1910) proposed three subgenera for *Streptocephalus*: *Streptocephalellus* (containing *Streptocephalellus purcelli* and *Streptocephalellus papillatus*); *Streptocephalus* (containing *Streptocephalus dichotomus* and *Streptocephalus lamellifer*) and *Streptocephalopsis* (containing *Streptocephalopsis rothschildi* and *Streptocephalopsis cafer*). Barnard (1929) rejected Daday's (1910) subgeneric divisions, as did Linder (1941). Barnard (1929) was the first author to altogether abandon the use of subgenera in *Streptocephalus*. More recently however, Brendonck, Hamer & Thiery (1992) designated a new subgenus, *Parastreptocephalus* (containing *Streptocephalus lamellifer*, *Streptocephalus sudanicus*, *Streptocephalus kaokoensis* and *Streptocephalus zuluensis*). The single Australian species, *Streptocephalus queenslandicus* recently described by Herbert & Timms (2000) has also been included in the latter subgenus. The validity of none of these subgeneric divisions has been tested previously rendering their utility dubious.

The present study has two objectives. First, to examine the phylogenetic relationships within *Streptocephalus* and to determine the biogeographical origin of the genus. Second, to test the validity of the morphological groups defined by Maeda-Martinez *et al.* (1995a) and subgenera defined by earlier taxonomists. The data presented in this study represents the first molecular phylogeny for *Streptocephalus* and provides a useful framework to examine the biogeographical origin of other freshwater invertebrate groups, as similar large-scale studies are central in determining the historical relationships between continental faunas.

MATERIAL AND METHODS

SAMPLE COLLECTION

Animals were collected from ephemeral pools or hatched from soil samples identified and preserved in either 70% or absolute alcohol until used in DNA extraction. A list of the 23 species sampled and examined in the present study is provided in Table 1. A large number of species had been collected only from the type locality and were formalin fixed, rendering these samples unavailable for inclusion in the present

study. Sampling was undertaken in such a manner that the major biogeographical regions were incorporated into the present study, except for India, as no samples could be secured from this region. Where possible, multiple representatives from different localities were collected. Voucher specimens, where available from this present study were deposited in the South African Museum, Cape Town, South Africa (accession numbers in Table 1).

DNA EXTRACTION, PCR AND SEQUENCING

Total genomic DNA was extracted from whole animals using the phenol/chloroform: isoamyl alcohol method outlined in Hillis, Moritz & Mable, (1996). DNA concentrations were determined spectrophotometrically and samples diluted to a final concentration of 40 ng/μL. For the 12S rRNA mitochondrial DNA (mtDNA) data set multiple samples from different geographical localities were used for some species when material was available. For the cytochrome oxidase subunit I (COI mtDNA) data set a single representative of each species was amplified and used in the phylogenetic reconstruction. Both of these mtDNA genes have been successfully used in evolutionary studies in a wide range of crustaceans, including other anostracans (Hanner & Fugate, 1992; Schubart, Diesel & Hedges, 1998; Remigio & Hebert, 2000; Remigio, Hebert & Savage, 2001) whereas the ITS-1 region has been used in a number of recent studies on crustaceans to explore its phylogenetic utility (Harris & Crandall, 2000; Rocha-Olivares, Fleeger & Foltz, 2001). A subset of the data (Table 1) was used to examine the utility of the nuclear ITS-1 region in recovering phylogenetic relationships among the North American taxa.

The primers 12Sa and 12Sb (Kocher *et al.*, 1989) were used to amplify the 12S rRNA mtDNA. The primers LCO-1490 and HCO-2198 (Folmer *et al.*, 1994) were used to amplify the COI mtDNA. For the ITS-1 region the primers 5.8 A and HI (Hillis & Dixon, 1991) were used to amplify the 5.8S region, the entire ITS-1 and a fragment of the 18S genome a subset of the original samples were used to investigate the utility of this marker. PCR was performed using the protocol detailed in Daniels *et al.* (2002). PCR products were purified using a QIA quick gel purification kit (QIAGEN). These purified products were cycle sequenced using Big Dye version 3 following the recommendations of the manufacturer (Applied Biosystems, ABI). Unincorporated dideoxynucleotides were removed using Sephadex G-25 (Sigma) filtration and the reactions were electrophoresed on an ABI 3100 PRISM Genetic Analyser (ABI). For some species, only one gene fragment could be successfully amplified (*Streptocephalus woottoni* and *Streptocephalus dregei*,

only 12S rRNA could be amplified; *Streptocephalus cladophorus* – only COI could be amplified). The absent gene regions were coded as missing data in the combined mtDNA analysis.

OUTGROUP SELECTION

Two recent molecular studies (Remigio & Hebert, 2000; Spears & Abele, 2000) have suggested that the family Thamnocephalidae is the sister group of Streptocephalidae. Both of these studies, however, suffered from limited sampling of all the Anostraca families. In the present study, *Thamnocephalus platyurus* was selected as an outgroup. The use of the latter outgroup did not reveal a topology that was different when any of the additional more distantly related outgroups were used.

PHYLOGENETIC ANALYSIS

To improve accuracy, both heavy and light strands were sequenced and consensus sequences created. Base ambiguities were corrected using Sequence Navigator (Applied Biosystems). The 12s rRNA and ITS-1 sequence data were aligned in CLUSTAL W version 1.6 (Thompson, Higgins & Gibson, 1994) using the default parameters and manually adjusted by eye, while the COI sequences was aligned manually. All sequences from this study have been deposited in GenBank. Accession numbers are: for 12S rRNA mtDNA, AY519779–AY519816; for COI mtDNA, AY519817–AY519838; and for the nuclear fragment, AY519839–AY519851.

Data analyses were executed in PAUP*4.0 b10 (Swofford, 2002) using maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) methods. For the MP analysis, trees were generated using the heuristic search option with TBR branch swapping and 1000 random taxon additions. Recently, ML analysis has become particularly favoured as it permits the application of evolutionary models resulting in a more powerful phylogenetic inference (Posada & Crandall, 2001). For the ML analyses the appropriate substitution model was selected using MODELTEST version 3.06 (Posada & Crandall, 1998). The optimal parameters of the ML models for the sequence data sets were incorporated in the calculation of sequence divergence values. For the combined sequence data MP and ML analyses were performed, for the latter analysis MODELTEST was again used to determine the substitution model. The best-fit model was then incorporated into ML and a heuristic search with branch swapping was performed. Confidence in the nodes recovered by MP was estimated by performing 1000 bootstrap replicates (Felsenstein, 1985). Due to time constraints only 100 bootstrap replicates were

Table 1. *Streptocephalus* species sampled in the present study are listed in alphabetical order. Where available, the date, collector's name and the geographical positions are provided (12S = 12S rRNA mtDNA). Museum accession numbers (SAM, South African Museum, Cape Town, South Africa) are also provided

Species	Country	Date collected	Collector	Locality	GPS co-ordinates	Gene
<i>S. bourquinii</i>	South Africa	–	M. Hamer	KwaZulu-Natal, False Bay Park in the St Lucia area	27°55' S, 32°20' E	12S and COI
<i>S. boumani</i> (SAM A45173)	USA	18 September 2000	D. C. Rogers	Cochise, Arizona, Wilcox Playa	–	12S, COI and nuclear fragment
<i>S. cafer</i>	South Africa	–	M. Hamer	North-West Province, Batsalono	–	12S and COI
<i>S. cladophorus</i>	Namibia	–	M. Hamer	Oshivanda	–	COI
<i>S. dorotheae</i> 1	USA	20 July 2000	B. Lang & D. C. Rogers	Eddy County, Lin National Forest, Queens Quad	–	12S, COI and nuclear fragment
<i>S. dorotheae</i> 2	Mexico	–	A. Maeda-Martinez	Rancho Balmaceda ranch – Cabo San Lucas, Los Cabos, Baja California Sur	–	12S
<i>S. dumonti</i>	USA	23 August 2000	B. Lang & D. C. Rogers	Cibola County, Zuni Mountains, New Mexico	–	12S, COI and nuclear fragment
<i>S. guzmanii</i> (SAM A45177)	Mexico	3 July 2001	A. Maeda-Martinez	Saucillio, Veisca, Coahuila	25°26' 04" N 102°56' 04" W	12S, COI and nuclear fragment
<i>S. mackini</i> 1 (SAM A45172)	USA	19 September 2001	B. Lang & D. C. Rogers	Luna County Pony Hills – Tank	–	12S, COI and nuclear fragment
<i>S. mackini</i> 2 (SAM A45179)	Mexico	–	A. Maeda-Martinez	10.3 km E Ciudad del Maiz, Ciudad del Maiz	22°26' 33" N 99°38' 24" W	12S
<i>S. macrouris</i>	Namibia	–	M. Hamer	–	–	12S and COI
<i>S. moorei</i>	USA	20 September 2000	D. C. Rogers & B. Lang	Luna County, East Columbus, Stock tank	–	12S, COI and nuclear fragment
<i>S. namibiensis</i> 3 (SAM A45178)	South Africa	2 August 2002	A. Abrahams (raised from soil samples)	Northern Cape Kimberley area, Holpan	28°39' 53" S 24°27' 45" E	12S
<i>S. namibiensis</i> 2 (SAM A45170)	South Africa	2 August 2002	A. Abrahams (raised from soil samples)	Northern Cape Kimberley area, Platfontein	28°40' 05" S 24°37' 05" E	12S
<i>S. namibiensis</i> 1 (SAM A45169)	South Africa	–	M. Hamer	Northern Cape Vaalbos Game Reserve	–	12S, COI and nuclear fragment
<i>S. ovamboensis</i>	South Africa	–	M. Hamer	Northern Cape Tankwa, Karoo National Park	–	12S and COI
<i>S. papillatus</i>	South Africa	–	M. Hamer	Northern Cape Kenhart area	–	12S, COI and nuclear fragment
<i>S. proboscideus</i> (SAM A45182)	South Africa	–	A. Rosa	Northern Cape Farm – Alpha, mid Kalahari; 35 km south of Gembok Park	–	12S, COI and nuclear fragment
<i>S. purcelli</i> 1 (SAM A45176)	South Africa	–	M. Hamer	Western Cape Ysterfontein	33°20' S 18°20' E	12S, COI and nuclear fragment

<i>S. purcelli</i> 2 (SAM A45186)	South Africa	29 August 2002	M. Norval	Northern Cape Namaqua National Park, Kammisskroon	30°25' S 17°17' E	12S
<i>S. purcelli</i> 3	South Africa	29 August 2002	M. Norval	Northern Cape Namaqua National Park, Kammisskroon	30°25' S 17°17' E	12S
<i>S. queenslandicus</i>	Australia	November 2002	B. Herbert	Walkamin, via Atherton, Northern Queensland	17°08' S 145°26' E	12S and COI
<i>S. sealii</i>	USA	7 June 2002	M. Belk	9 miles NE on highway; 57–7.1 miles SW of junction of 481 and 57	–	12S, COI and nuclear fragment
<i>S. sealii</i>	USA	1 August 2001	T. Spears	Munson Sand Hills (MSH 18); Apalachicola National Forest, Florida	30°21' 01" N 084°17' 05" W	12S
<i>S. sealii</i>	USA	28 August 2001	T. Spears	Munson Sand Hills (MSH 244); Apalachicola National Forest, Florida	30°21' 51" N 084°19' 36" W	12S
<i>S. sealii</i>	USA	4 September 2001	T. Spears	Munson Sand Hills (MSH XXXI); Apalachicola National Forest, Florida	30°21' 01" N 84°19' 31" W	12S
<i>S. sealii</i>	USA	29 August 2001	T. Spears	Munson Sand Hills (MSH 180); Apalachicola National Forest, Florida	30°20' 51" N 084°17' 09" W	12S
<i>S. sealii</i> (SAM A45185)	USA	13 July 2002	D. C. Rogers	Carson Spur, Amador County, California	–	12S
<i>S. sealii</i>	USA	7 June 2002	M. Belk	Maverick County, Texas; 8 miles south-west of 481 and 57	–	12S
<i>S. sudanicus</i> (SAM A45171)	Senegal	25 October 2002	M. Sarr	Kouthioum	–	12S and COI
<i>S. texanus</i> 1 (SAM A45181)	USA	22 August 2001	B. Lang & D. C. Rogers	Cibola County, Cibola National Forest, New Mexico	–	12S, COI and nuclear fragment
<i>S. texanus</i> 2 (SAM A45174)	USA	28 April 2002	M. Belk	1.2 miles west of Texas Hwy 173 on Co 241	–	12S
<i>S. texanus</i> 3	Mexico	–	A. Maeda-Martinez	0.5 km NW of Vizcaino; Federal Highway – Mulege, Baja California	27°39' 33" N 113°23' 23" W	12S
<i>S. torvicornis</i> * (SAM A45187)	Europe (Belgium)	January 2003	L. Bejdahl	Laboratory culture	–	12S and COI
<i>S. trifidus</i>	Zambia	–	M. Hamer	Livingstone	–	12S, COI and nuclear fragment
<i>S. woottoni</i> 1	USA	9 April 1998	T. Bomkamp**	California, Orange County; 150 ft north of El Toro in SCE easement	33°40' 00" N 117°38' 30" W	12S
<i>S. woottoni</i> 2	USA	16 March 1998	T. Bomkamp**	California, Ventura County, City of Moorpark-pool; near intersection of 23 Freeway and Tierra Rejada	34°16' 00" N 118°52' 30" W	12S

**Streptocephalus torvicornis* is a widespread species that occurs in North Africa, Europe and Asia, the sample in the present study is from an unknown locality. Systematic boundaries in this species need investigation. **Samples donated by the Los Angeles County Museum – LACM CR 981061.

performed for ML. In this study, we regard bootstrap values <60% as poorly supported nodes, bootstrap values between 60 and 70% as weakly supported and values >70% as well supported. The sequence data for the two mitochondrial genes were combined, following a partition homogeneity test (Farris *et al.*, 1995), however, irrespective of the outcome the 12S rRNA and COI mtDNA data sets were combined as both genes are in effect linked on the mitochondrial genome. The nuclear markers were not combined with the two mitochondrial gene fragments due to the limited number of taxa sequenced.

TESTING SUBGENERA AND ALTERNATIVE TOPOLOGIES ON THE COMBINED mtDNA DATA

The likelihood of topologies constrained to reflect the monophyly of the subgenera and the morphological groups proposed by Maeda-Martinez *et al.* (1995a) were statistically compared to the unconstrained topologies the present study using the Shimodaira & Hasegawa (1999) test as implemented in PAUP*4.0 b10 (Swofford, 2002). In an attempt to test Bănărescu's (1990) hypothesis of a recent dispersal event from Laurasia into North America and Australia, we constrained all of these species into a monophyletic group to test this recent dispersal hypothesis using the Shimodaira & Hasegawa (1999) test.

RESULTS

12S rRNA SEQUENCE TOPOLOGY

A 314 base pair fragment of the 12S rRNA gene was amplified. For the 12S rRNA data set, the GTR (Rodriguez *et al.*, 1990) + I + Γ was selected as the appropriate substitution model using MODELTEST (-lnL = 2710.11), with the following parameters, the base frequency of A = 0.3387, C = 0.0936, G = 0.1614 and T = 0.4062; rate matrix was $R_1 = 0.6679$, $R_2 = 6.6502$, $R_3 = 1.0078$, $R_4 = 0.6981$, $R_5 = 14.6381$ and $R_6 = 1.0000$, with the proportion of invariable sites (I) of 0.4699 and gamma correction of (α) = 0.4698. Resolution within the ML tree (Fig. 1) was moderate. The North American taxa fell into a poorly supported monophyletic clade (<60% bootstrap support). Within this clade, the relationship between *Streptocephalus dumonti* and *Streptocephalus texanus* was supported (70%). In addition, a close phylogenetic relationship between *Streptocephalus dorotheae* and *Streptocephalus moorei* was also recovered (77% bootstrap support). Among conspecific samples a close phylogenetic relationship was always recovered. For example, the relationship between *S. woottoni* populations was recovered with 100%, while the relationship between *Streptocephalus mac-*

kini and *S. texanus* was recovered with 77% and 62%, respectively. However, within *Streptocephalus sealii* two divergent clades were evident, comprising of samples from Florida (subclade 1 – with 68% bootstrap support) to samples from California and Texas (subclade 2 – with 86% bootstrap support). Among the African taxa sampled, the sister-taxa relationship between *Streptocephalus namibiensis* and *Streptocephalus proboscideus* was supported with 72% bootstrap support. The resolution using this marker was restricted to the terminal branches, with multiple representatives of species generally forming strongly supported clades. The parsimony analysis included 99 informative characters and recovered four trees, with a tree length of 540 steps (consistency index) CI = 0.35 and (retention index) RI = 0.62. The bootstrapped MP tree also recovered the monophyly of the North American species, with 65% support. Within this clade, most terminal relationships were well resolved. For example, within the *S. sealii* clade two distinct clades were evident, Florida (subclade 1) and Texas and California (subclade 2), with 95% and 81% bootstrap support, respectively. The close phylogenetic relationship was also recovered between *S. dumonti* and *S. texanus* (84%). As with the ML tree the sister taxa relationship between *S. namibiensis* and *S. proboscideus* was affirmed with 90% bootstrap support.

The sequence divergence values calculated using the ML model parameters demonstrated pronounced levels of genetic differentiation. Corrected intraspecific sequence divergence values ranged from as low as 0.31% between *S. woottoni* samples, 0.90% between *S. namibiensis* samples, 9.32% between *S. texanus* samples, 11.49% between *S. purcelli* samples, 12.02% between *S. mackini* samples, 13.07% between *S. dorotheae* samples to as high as 19.69% between the two divergent *S. sealii* groups comprising Florida, California and Texas. The results suggest that most species for which multiple representatives were collected may be comprised of genetically highly divergent evolutionary lineages. Interspecific corrected sequence divergence values were generally pronounced, and ranged from as low as 5.20% between *S. namibiensis* and *S. proboscideus*, 0.30% between *S. dumonti* and *S. texanus*, to as high as 75.70% between *S. guzmani* and *S. dregei*. Generally, the corrected interspecific sequence divergence values were between 20 and 50%.

COI SEQUENCES (TOPOLOGY NOT SHOWN)

A 599 base pair fragment was amplified. For the COI data set, the GTR (Rodriguez *et al.*, 1990) + Γ model was also selected using MODELTEST (-lnL = 3989.53), with the following base frequencies, A = 0.2850, C = 0.1785, G = 0.0817 and T = 0.4548;

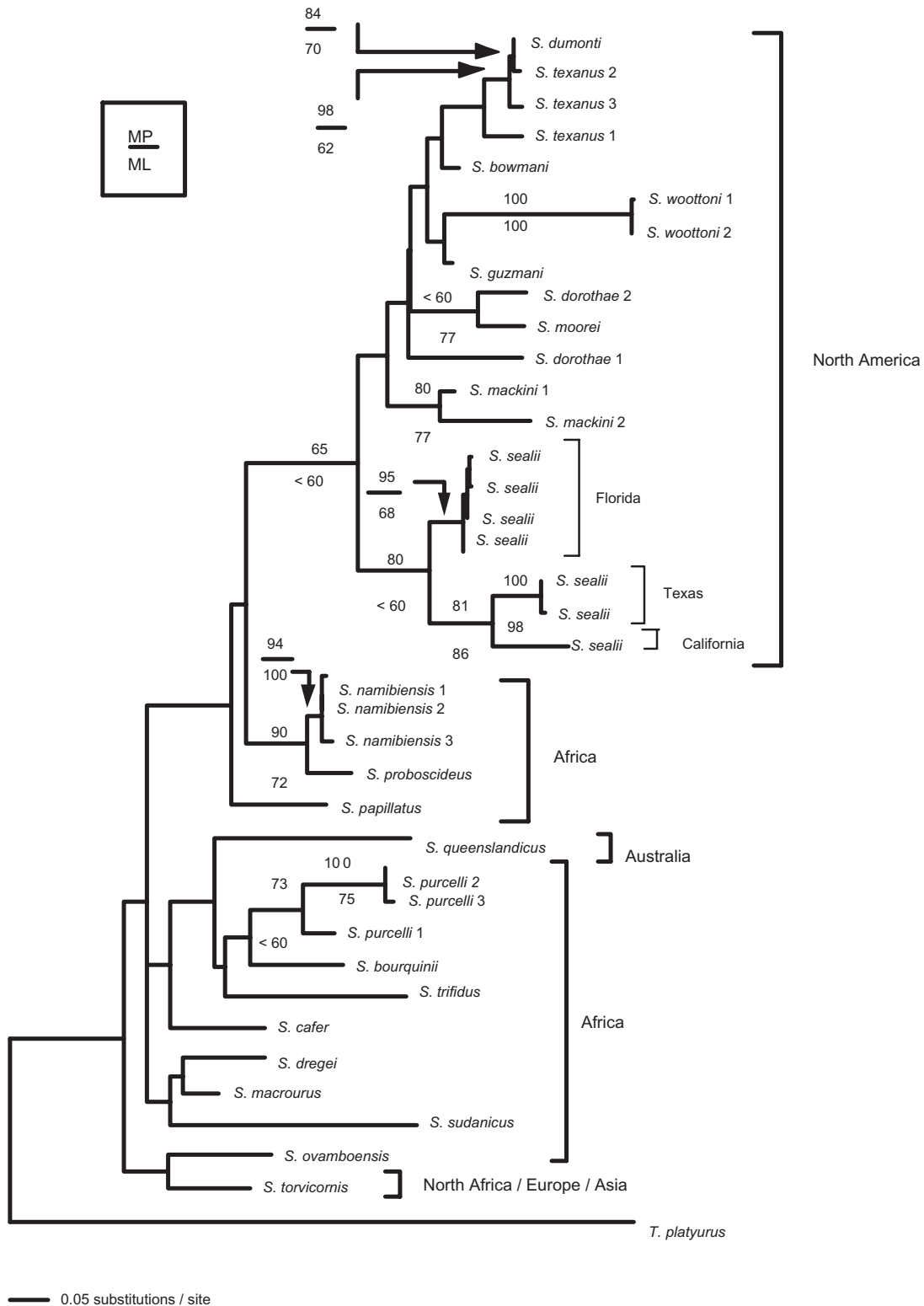


Figure 1. A maximum likelihood (ML) tree derived from the 12S rRNA mtDNA sequence data incorporating the GTR (Rodriguez *et al.*, 1990) + I + Γ model ($-\ln L = 2710.11$), with bootstrap values above the nodes for maximum parsimony (MP) 1000 pseudoreplicates and below the nodes for ML 100 pseudoreplicates. Bootstrap values $<60\%$ are not shown. *Streptocephalus torvicornis* is a widespread species, present in North Africa, Asia and Europe.

the rate matrix was $R_1 = 0.00001$, $R_2 = 9.6165$, $R_3 = 0.6259$, $R_4 = 0.4817$, $R_5 = 4.8732$ and $R_6 = 1.0000$, with $\alpha = 1.2602$. The monophyly of the North American samples were well supported in the ML analysis (bootstrap support of 68%). Among the North American species, a sister taxa relationship between *S. moorei* and *S. mackini* was recovered (76%), while the sister taxa relationship between *S. guzmani* and *Streptocephalus bowmani* was supported (68%). Among the African species, the sister taxon relationship between *S. namibiensis* and *S. proboscideus* was supported (97%), as well as the sister taxon relationship between *S. purcelli* and *S. cladophorus* (100%). The parsimony analysis included 225 informative characters and recovered a single tree, with a tree length of 1114 steps, CI = 0.35, and RI = 0.36. The bootstrapped parsimony tree did not recover the monophyly of the North American species. However, it recovered the sister taxa relationships between *S. namibiensis* and *S. proboscideus* (99%) as well as the sister taxon relationship between *S. purcelli* and *S. cladophorus* (100%); *S. dumonti* and *S. texanus* (63%). Corrected interspecific sequence divergence values were generally high and ranged from 15.61% between *S. namibiensis* and *S. proboscideus*, to 15.89% between *S. dumonti* and *S. texanus*, generally the corrected sequence divergence varied between 25 and 75%.

COMBINED mtDNA SEQUENCE DATA (12S rRNA AND COI mtDNA)

The results from the partition homogeneity test showed that the two data sets were largely congruent ($P = 0.87$), allowing for the combination of the data sets. For the combined data set the best-fit substitution model was also GTR (Rodriguez *et al.*, 1990) + I + Γ ($-1 \text{ nL} = 7574.18$; Fig. 2), the base frequencies

were as follows, A = 0.3224; C = 0.1354, G = 0.1379 and T = 0.4042, the rate matrix was $R_1 = 0.2884$, $R_2 = 7.2146$, $R_3 = 1.2379$, $R_4 = 0.9843$, $R_5 = 14.0003$ and $R_6 = 1.0000$, with I = 0.4851 and $\alpha = 0.5505$. The monophyly of the North American taxa was recovered with 86% bootstrap support, similarly the sister taxa relationships between *S. namibiensis* and *S. proboscideus*, and *S. purcelli* and *S. cladophorus* were well supported (>95% bootstrap support). The parsimony analysis of the combined data sets contained 298 parsimony informative characters and recovered a single tree with a tree length of 1552 steps, CI = 0.35 and RI = 0.38. The monophyletic relationship among the North American species was reaffirmed with 78% bootstrap support. Within this clade, the sister taxa relationship between *S. texanus* and *S. dumonti* was supported with 68% bootstrap support. As with the COI data, *S. namibiensis* was the sister-taxon of *S. proboscideus* with *S. papillatus* the sister taxa of these two species, while *S. purcelli* was the sister taxon of *S. cladophorus* with 99% bootstrap support.

TOPOLOGICAL CONSTRAINTS FOR THE MITOCHONDRIAL MARKERS (FOR ALL OF THE SPECIES SAMPLED)

A summary of the results of the enforced constraint Shimodaira & Hasegawa (1999) tests is presented in Table 2. All of the proposed morphological groupings with the exception of the Cafer group gave a topology that was statistically worse than the unconstrained ML tree. Similarly, all of the three subgeneric divisions tested recovered a topology that was statistically worse. In addition, enforcing a Laurasian origin for the North American taxa recovered a topology that was statistically worse than the best tree, thus invalidating Bănărescu's (1990) recent dispersal hypothesis.

Table 2. Results of the Shimodaira & Hasegawa (1999) test for the proposed morphological groupings, suggested by Maeda-Martinez *et al.* (1995a), and subgenera, based on a maximum likelihood analysis of the combined 12S rRNA and COI mtDNA data. The species that were constrained are given in parentheses

Topology tested	-1 nL	$\Delta -1 \text{ nL}$	P
Unconstrained tree (12S rRNA and COI)	7544.44		
Sealii group (<i>S. sealii</i> , <i>S. bourquinii</i> and <i>S. woottoni</i>)	7588.08	43.63	0.000
Cafer group (<i>S. cafer</i> and <i>S. trifidus</i>)	7547.35	2.91	0.235
Proboscideus subgroup (<i>S. cladophorus</i> , <i>S. namibiensis</i> and <i>S. proboscideus</i>)	7735.02	190.57	0.000
Torvicornis subgroup (<i>S. ovamboensis</i> , <i>S. torvicornis</i> and <i>S. guzmani</i>)	7714.64	170.19	0.000
Mackini group (<i>S. dorotheae</i> , <i>S. mackini</i> , <i>S. moorei</i> and <i>S. texanus</i>)	7570.88	26.43	0.007
(Daday, 1910) (<i>S. purcelli</i> and <i>S. papillatus</i>)	7719.63	175.18	0.000
<i>Parastreptocephalus</i> (<i>S. sudanicus</i> and <i>S. queenslandicus</i>)	7602.06	57.62	0.000
Laurasian dispersal – (Bănărescu, 1990) <i>S. torvicornis</i> sister to <i>S. queenslandicus</i> *	7700.58	156.13	0.000

*sister taxa to all North American taxa.

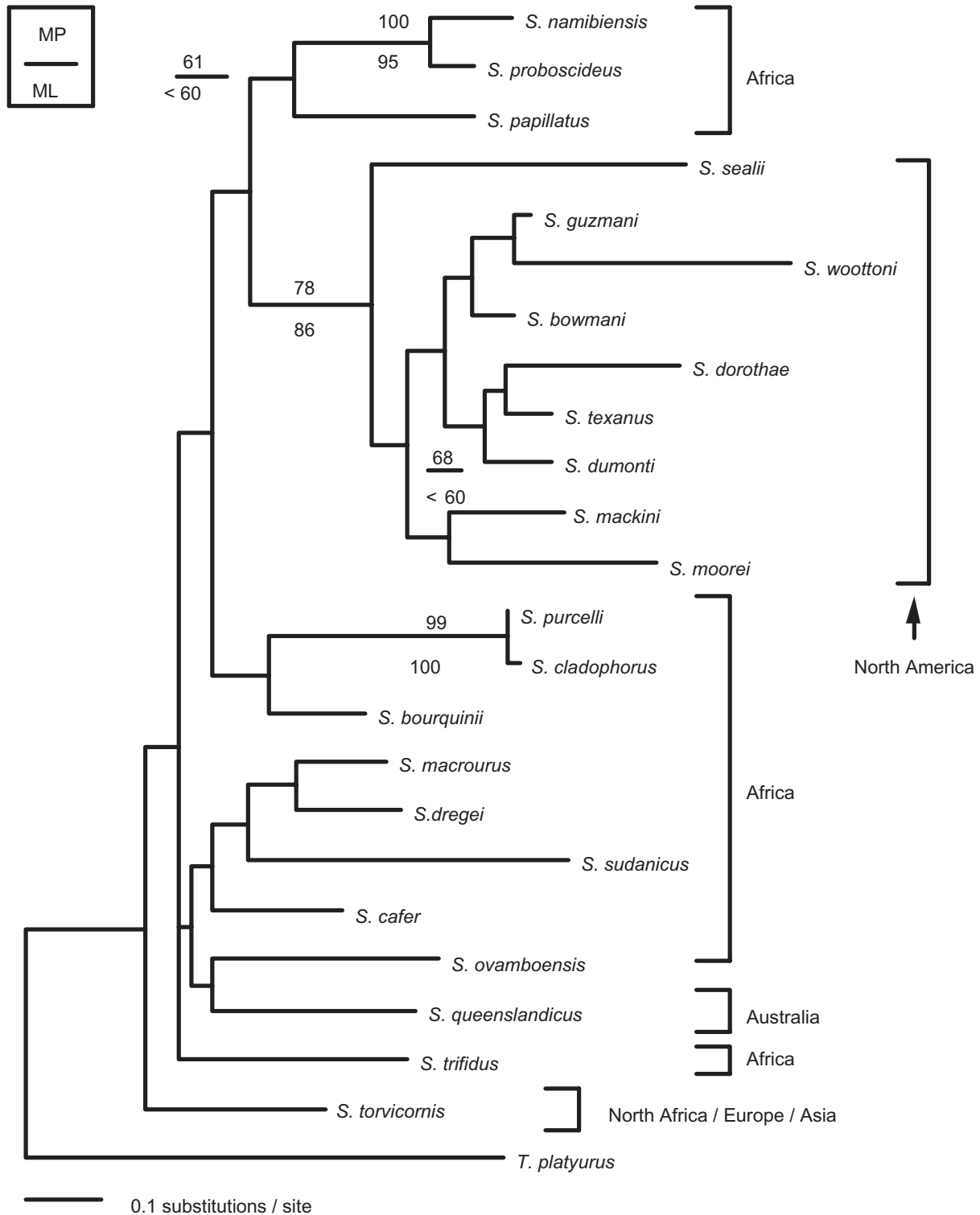


Figure 2. Total mtDNA (12S rRNA and COI mtDNA) evidence maximum likelihood (ML) tree, incorporating the GTR + I + Γ model ($-\ln L = 7574.18$) with bootstrap values above the nodes for maximum parsimony and below the nodes for ML. Bootstrap values <60% are not shown.

NUCLEAR SEQUENCE TOPOLOGY (5.8S-ITS-1-18S)

The nuclear fragment amplified was variable in size and ranged from 1000 base pairs (bp) to greater than 1500 bp. A similar length fragment (between 760 and 1300 bp) was reported for the freshwater crayfish genera *Orconectes* and *Procambarus* (Harris & Crandall, 2000). The best-fit substitution model for the nuclear DNA data set was HKY 85 (Hasegawa, Kishino & Yano, 1985) + γ ($-1 \text{ nL} = 7139.91$; Fig. 3), the base frequencies were as follows, A = 0.2041, C = 0.3300, G = 0.2779 and T = 0.1879 with a transition/transversion (ti/tv) ratio of 1.0428 and $\alpha = 3.9654$. The ML topology obtained with the combined mitochondrial sequence data was remarkably similar to the nuclear tree topology. The nuclear topology recovered the relationships between *S. moorei* and *S. dorotheae* (the latter two species being sister taxa) with high bootstrap support (100%), *S. mackini* was the sister taxa to these two species (72% support). Similarly, the sister taxa relationship between *S. guzmani* and *S. bowmani* (with 96% support) and *S. texanus* and *S. dumonti* (100%) were well supported. The monophyly of the North American species was not recovered. A Shimodaira & Hasegawa (1999) test was performed on the nuclear tree to enforce the monophyly of the North American clade (as detected with the two mitochondrial markers, Fig. 2), and recovered a statistically worse topology when compared with the best ML topology ($\Delta -1 \text{ nL} = 19.88$; $P = 0.003$).

The four African species sequenced (*S. papillatus*, *S. proboscideus*, *S. namibiensis* and *S. purcelli*) fell into a monophyletic clade (94% bootstrap support). Similar to the combined mtDNA data, the relationship between *S. proboscideus* and *S. namibiensis* is well resolved (100% bootstrap support) with *S. papillatus* being the sister taxa to these two species (94% bootstrap support). The parsimony analysis contained 504 informative characters. The parsimony analysis recovered a single tree length of 1878 steps, CI = 0.67 and RI = 0.63. Bootstrap support for the parsimony analysis supported the same phylogenetic relationships as evident in the ML topology, with bootstrap values >92%.

Interspecific sequence values were high between species from the two continental areas: Africa and North America. Among the four African species, the corrected sequence divergence values ranged from as low as 0.90% between *S. namibiensis* and *S. proboscideus* while the corrected sequence divergence values between the North American species were exceptionally high (>90%).

DISCUSSION

The combined mtDNA data presented in this study demonstrate that all of the North American species

fall into a well-supported monophyletic clade and that none of these species have a close phylogenetic relationship with species from Africa and Europe as proposed by Maeda-Martinez *et al.* (1995b). The North American taxa were not the sister taxa to the Australian or Eurasian species as would be expected if the current distribution patterns were the result of a single dispersal from Asia into North American and Australia. These results are in direct contrast to the hypotheses of Wiman (1979), Belk (1984) and Bănărescu (1990) and support the hypothesis of Maeda-Martinez *et al.* (1995a, b), indicating that the current distribution patterns are probably best explained through vicariance events during the fragmentation of Gondwana. However, considering the limited number of species sequenced in the present analysis a solid conclusion on the biogeography of the group is not yet possible. The provisional data do however, support an ancient radiation within *Streptocephalus*. While the monophyly of the group is not well supported in the present study, a recent molecular study derived from 18S rRNA demonstrated the family is a well-supported monophyletic group (Weekers *et al.*, 2002). These results are further corroborated by the unique antennal morphology of the family.

Furthermore the results are in contrast to the cladistic analysis of Maeda-Martinez *et al.* (1995b), suggesting that the limited number of morphological characters used in their analysis can generally be regarded as homoplasious (with the exception of the Cafer group). Similarly, none of the morphological groups (based largely on male antennae and frontal appendages) proposed by Maeda-Martinez *et al.* (1995a) and Hamer *et al.* (1994a, b), or the proposed subgenera are systematically valid taxonomic units. Such false taxonomic grouping should be abandoned. The usefulness of morphology in the reconstruction of phylogenetic relationships, as well as for determining systematic affinities (e.g. subgenera) in *Streptocephalus* is limited due to the apparent large degree of convergent evolution that the group has undergone. Until further studies are conducted to determine more useful morphological characters, inferences derived from morphology should be regarded with caution. Such results have been reiterated by recent studies on the Anostraca (Remigio & Hebert, 2000; Remigio, Herbert & Savage, 2001; Remigio, Timms & Herbert, 2003). For example, Remigio *et al.* (2001) reported that similarities in the reproductive structures (brood pouch and penial morphology) reflected convergent evolution among *Parartemia* species. More recently Remigio *et al.* (2003) reported that the frontal appendages in *Branchinella* appear to be subject to considerable homoplasy.

Using the molecular clock of 0.5% per million years for 12S rRNA mtDNA as suggested by Lynch & Jarrel,

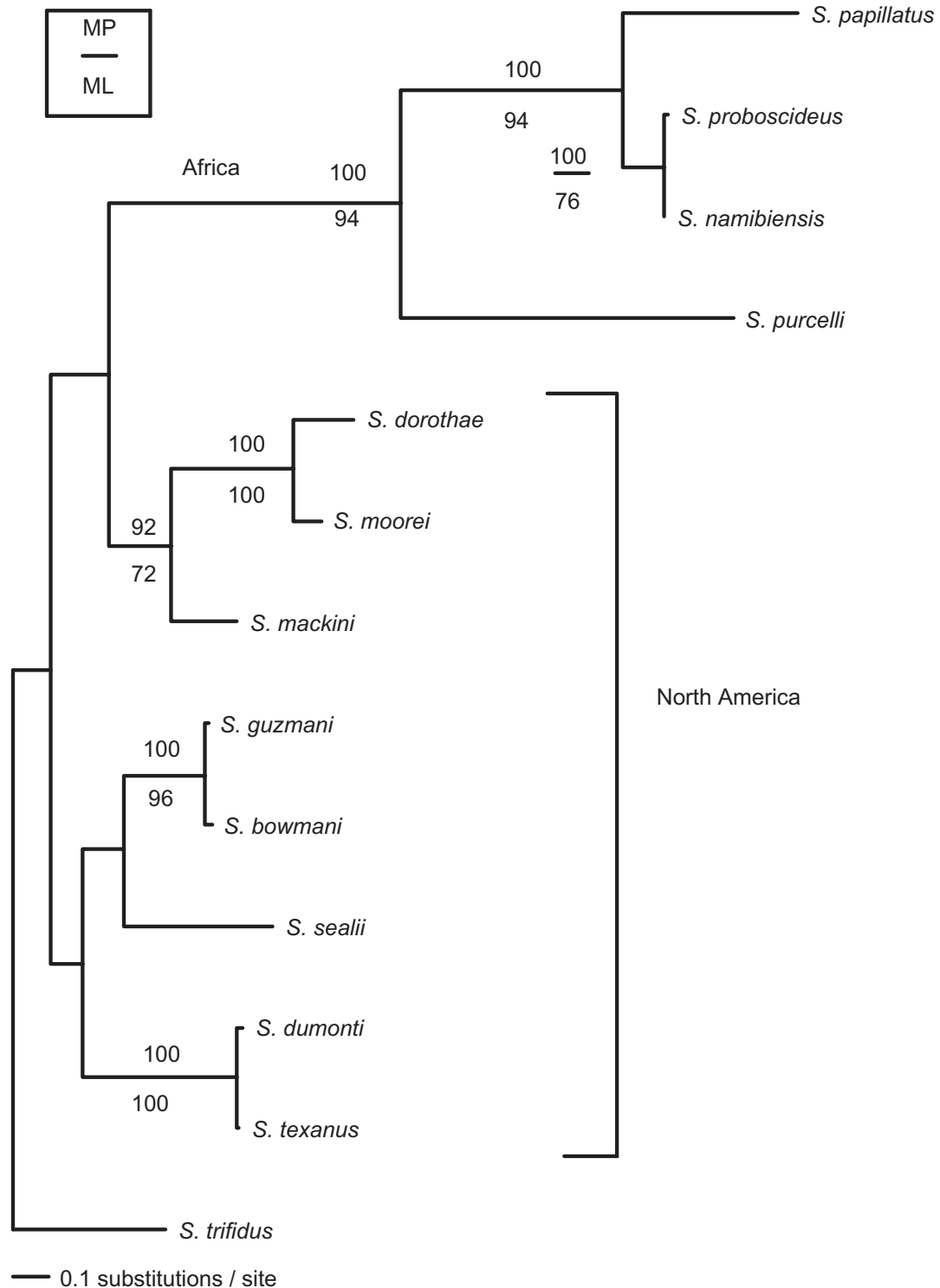


Figure 3. A maximum likelihood (ML) tree derived from the nuclear markers, incorporating the HKY 85 (Hasegawa, Kishino & Yano, 1985) + Γ model ($-\ln L = 7139.91$) with bootstrap values above the nodes for maximum parsimony 1000 pseudoreplicates; below the nodes for ML 100 pseudoreplicates. Bootstrap values <60% are not shown.

1993) and used by Colbourne & Hebert (1996) and the divergence of 1% per million years for COI as suggested by Knowlton *et al.* (1993) (for snapping shrimp, *Alpheus*) and Schubart *et al.* (1998) (for the grapsid crab genera *Sesarma* and *Metopaulias*), it appears that *Streptocephalus* evolved 162–180 Mya (based on the maximum corrected sequence divergence between taxa – values derived from 12S rRNA and COI mtDNA) during the early Jurassic. These clock calibrations may represent an underestimation as Hebert *et al.* (2002) demonstrated that freshwater anostracan lineages might have a reduced rate of molecular evolution when compared to halophilic anostracans. In the absence of fossil data for this genus with which to calibrate a molecular clock these divergence estimations should be treated with caution. Nevertheless, the application of these molecular clocks to the sequence data from the present study demonstrated that the genus was present in Africa and Australia during the early Jurassic. The post Jurassic epochs were characterized by marked continental fragmentation and drift due to plate tectonics that resulted in the subsequent isolation of continental landmasses. This period was also characterized by marked climatic oscillations, generally becoming much warmer and drier (Tyson, 1986), ideal environmental conditions for the diversification of this thermophilic genus. The basal placement of the African and Australian taxa in *Parastreptocephalus* indicates that the subgenus probably had an origin during a period of connectivity between Africa and Australia in Gondwana. Interestingly, the two species sampled in this subgenus (*S. (P.) sudanicus* and *S. (P.) queenslandicus*) are characterized by tetrahedral cysts that may have served a selective advantage in the dispersal in this group, as evident from the present widespread distribution of *S. (P.) sudanicus* (Brendonck *et al.*, 1992).

There is a noticeable faunistic link for a number of Gondwanan freshwater crustacean taxa between Africa and Australia and other continental landmasses such as India (Newman, 1991). For example, the presence of the fairy shrimp genera *Streptocephalus* and *Branchinella*, freshwater phreatoicidean isopods (Amphisopodidae), amphipods of the family Paramalitidae and the Spelaeogriphacea in Africa and Australia, with distinct elements (either extant or extinct) present in Asia, Europe, North America and India all point to the antiquity of these freshwater crustacean groups (Poore & Humphreys, 1998; Wilson & Johnson, 1999). In this respect, molecular phylogenetic studies on the latter groups may be useful in determining their radiation and provide new insight into the evolution of continental freshwater crustacean assemblages.

The fact that all of the North American species have a monophyletic origin suggests that either a single dis-

persal or a single vicariant event led to the formation of this fauna. The low species diversity in North America is problematic to explain. However, given the antiquity of species (as evident from the marked sequence divergence values and the long branches) on this continent, it is likely that the fauna have been severely affected by glaciation events and that the colder temperatures in the Northern Hemisphere may have prevented marked speciation in this normally thermophilic group. For example, Hewitt (2000) demonstrates that during the Quaternary (2.4 Mya) the ice sheets that covered North America advanced as far south as 40°N. The low species diversity of *Streptocephalus* in Australia is indeed very interesting, and may reflect the inability of this genus to compete with species of *Branchinella*, which is the dominant genus in Australia.

Phylogenetic resolution was largely restricted to the terminal branches, while the internal branches were generally poorly resolved. Of the two gene fragments sequenced, the 12S rRNA data provided more phylogenetic signals than the COI data. Although the latter gene contained a higher number of parsimony informative characters, it also contained the highest level of homoplasy. Similar results were reported by Remigio & Hebert (2000). While the resolution with COI was low, the topology obtained was remarkably congruent with the 12S rRNA tree. These results would suggest that COI is probably of limited utility in recovering phylogenetic relationships in *Streptocephalus*. Among the endemic Australian genus *Parartemia*, interspecific sequence divergence values for 16S rRNA ranged from 11.0 to 22.4%, while for the COI fragment sequence divergence values for *Parartemia* and *Branchinella* species were generally >15%. Corrected interspecific divergence values reported in the present study for the 12S rRNA and COI mtDNA gene were exceptionally high, pointing to the antiquity of *Streptocephalus*. Similarly, the nuclear gene fragments (5.8S-ITS-1-18S) exhibited marked levels of sequence divergence between *Streptocephalus* species. The pronounced sequence divergence values for the nuclear markers are among the highest reported to date, and may be attributed to the best-fit model employed. The ITS-1 gene region was informative at detecting a relationship between closely related species, however, it did not recover deeper nodes in the tree. The apparent incongruence between the mitochondrial and nuclear markers could possibly be attributed to the different modes of evolution between the markers, as well as the limited data set used in the construction of the nuclear phylogeny.

From the high intraspecific sequence divergence values, it can be inferred that gene flow between conspecific *Streptocephalus* populations is limited, further corroborating the inability of these organisms for

widespread dispersal as suggested by Bănărescu (1990).

For example, a corrected sequence divergence of 11.49% for 12S between the two conspecific populations of *S. purcelli* (Ysterfontein and Kammieskroom) collected in South Africa and separated by 340 km, suggests limited dispersal capacity. Similarly, the marked sequence divergence values (19.69%) between *S. sealii* populations are indicative of limited gene flow between conspecific populations. These results are rather surprising, considering that the Anostraca produce diapausing cysts that are well adapted to heat and drought stress. However, Remigio & Hebert (2000) concluded that avian vectors might also occasionally act as dispersal agents for these branchiopods. Results from the present study confirm earlier speculation by Brendonck, DeMeester & Riddoch (2000b), Brendonck *et al.* (2000a), and Brendonck & Riddoch (1999) that limited dispersal is probably occurring between distantly located populations of this fairy shrimp as also evident in the genus *Branchipodopsis*. This appears to be a general occurrence among conspecific branchiopod taxa. For example, in the tadpole shrimp *Lepidurus*, sequence divergence based on 12S rRNA mtDNA ranged from 0.3 to 3.4% between populations, while the sequence divergence among populations that are geographically isolated were as high as 12.6% (King & Hanner, 1998). These results indicate that a number of these morphologically similar streptocephalid species may comprise distinct genetic lineages (cryptic species), particularly in cases such as in *S. sealii*. King & Hanner (1999) came to the same conclusion while studying the phylogenetic relationships in *Lepidurus*. Similarly, Taylor, Finston & Herbert (1998) demonstrated the presence of cryptic and genetically divergent lineages among North American populations of the freshwater flea, *Daphnia laevis*. To this extent, detailed studies of intraspecific variation are needed to determine the magnitude of genetic variation and the systematic status of taxa, particularly for presumably widespread species. This study further highlights the need for the application of molecular markers in determining the biodiversity of anostracans. A more comprehensive geographical sampling regime is central for understanding the biogeography of this group. In addition, a future study could benefit from using more molecular markers in combination with morphological data to better resolve internal nodes in the phylogeny of *Streptocephalus*.

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