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Phylogenetic structure of the Sphaeriinae, a global clade of freshwater bivalve molluscs, inferred from nuclear (ITS-1) and mitochondrial (16S) ribosomal gene sequences

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The Sphaeriidae represent one of the primary molluscan radiations into freshwater environments. We have reconstructed phylogenetic relationships of the Sphaeriinae, a cosmopolitan sphaeriid subfamily, using variation in nuclear ribosomal first internal transcribed spacer (ITS-1) and mitochondrial large ribosomal subunit (16S) gene fragments. A total of 38 New World, Eurasian and Antipodean taxa were characterized, including all primary taxonomic groupings except for *Neopisidium*, and members of the sister clade Euperinae were employed as outgroups. Phylogenetic analyses of individual and combined (16S + ITS1) datasets all recovered a paraphyletic *Pisidium* and a derived clade of asynchronous brooding *Sphaerium/Musculium* taxa. Maximum parsimony as well as maximum likelihood analyses of combined data yielded largely congruent and well-resolved topologies, and robustly supported clades were utilized to revise current sphaeriine taxonomy. Instead of the traditionally accepted three cosmopolitan genera, *Pisidium s.l.*, *Musculium*, and *Sphaerium*, five major monophyletic lineages, *Afropisidium*, *Odhneripisidium*, *Pisidium*, *Cyclocalyx* and *Sphaerium*, were recognized at the generic level. In addition, a number of subgeneric level groups were recovered in *Sphaerium*: *Herringtonium*, *Sphaerium s.s.*, *Sphaerinova*, *Amesoda*, and *Musculium*, together with one unassigned species, *S. transversum*.

ADDITIONAL KEYWORDS: *Afropisidium* – *Odhneripisidium* – *Pisidium* – *Cyclocalyx* – *Sphaerium* – phylogeny – taxonomy.

INTRODUCTION

Although molluscs easily represent the most diverse segment of marine faunas (Brusca & Brusca, 1990), the number of major molluscan radiations into freshwater habitats is quite limited. Only three major groups, the basommatophoran pulmonate snails and the unionoidan and sphaeriid bivalves, have enjoyed a sufficiently long period of diversification in freshwater environments to produce taxonomically rich and globally distributed clades (Haas, 1969; Hubendick, 1979; Kuiper, 1983). Our study is concerned with one of these molluscan freshwater radiations, the Sphaeriidae, and aims to construct a comprehensive molecular phylogeny of one of its primary subgroupings: the cosmopolitan subfamily Sphaeriinae.

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The Sphaeriidae (fingernail/nut/pill/pea clams) are ubiquitous in freshwater ecosystems (Herrington, 1962; Clarke, 1973; Burch, 1975; Kuiper, 1983). They first appeared in the Cretaceous fossil record (Keen & Dance, 1969) but, at present, lack convincing marine outgroups (Dreher-Mansur & Meier-Brook, 2000; Park & Ó Foighil, 2000). Recent morphological (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002) and molecular studies (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002) distinguish two primary clades: the Sphaeriinae and the Gondwanan Euperinae. The Sphaeriinae contain three cosmopolitan genera, *Pisidium*, *Sphaerium* and *Musculium*, which have maximum diversities in the Holarctic (Ellis, 1962; Burch, 1975; Kuiper, 1983), and are diagnosed on the basis of details of the shell and soft-part morphology and of reproductive/developmental characteristics (Burch, 1975; Heard, 1977).

Sphaeriinids are often the dominant benthic organisms in streams and ponds (Avolizi, 1976; Eckblad *et al*., 1977) where they play a key role in energy and nutrient cycling (Alimov, 1970; Hornbach, Wissing & Burky, 1984; Holopainen & Hanski, 1986; Lopez & Holopainen, 1987; Way, 1988). They have arguably the most complicated pattern of parental care in the Bivalvia, involving extraoogonial nutrition of direct-developing young, incubated as either synchronous or asynchronous clutches, within ctenidial brood sacs (Gilmore, 1917; Okada, 1935; Heard, 1965, 1977; Mackie, Qadri & Clarke, 1974; Mackie, 1978; Hetzel, 1994). Sphaeriinids exhibit a remarkable degree of genome amplification (up to 13n) (Park, 1992; Baršienė, Tapia & Barsyte, 1996; Burch, Park & Chung, 1998; Lee, 1999) and a number of North American species may share ancestral genome duplication events which pre-date their cladogenesis (Lee & Ó Foighil, 2002).

Sphaeriinid systematics has historically been hampered by considerable ecophenotypic and allometric variation in shell shape (Holopainen & Kuiper, 1982; Bailey, Anthony & Mackie, 1983; Dyduch-Falniowska, 1983), a factor compounded by the extensive geographical ranges of many taxa. For instance, almost half of North American sphaeriids also occur in Eurasia (Herrington, 1962; Burch, 1975) and the nominal taxon *Pisidium casertanum* (Poli, 1791) is found on every continent except for Antarctica (Ellis, 1962; Herrington, 1962; Kuiper, 1983). Another problem has been the use of a strikingly divergent system of classification by the Russian taxonomic school (Scarlato & Starobogatov, 1979; Starobogatov, 1992; Korniushin, 1998c).

A number of sphaeriinid cladistic studies have recently been performed utilizing morphological (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002) and molecular (Cooley & Ó Foighil, 2000) datasets. Although all three morphological studies recovered a monophyletic *Pisidium*, the molecular dataset (mt 16S RNA sequences) yielded a paraphyletic *Pisidium* in which the subgenus *Afropisidium* was sister to all the other sphaeriine taxa considered. *Pisidium* paraphyly is also apparent in preliminary trees based on nuclear gene fragments: 28S ribosomal RNA (Park & Ó Foighil, 2000) and 6-phosphogluconate dehydrogenase (Lee & Ó Foighil, 2002). Another difference among these cladistic studies concerns the interrelationship of the synchronous (*Pisidium*) and asynchronous brooding (*Sphaerium* and *Musculium*) taxa. Two of the morphological analyses (Dreher-Mansur & Meier-Brook, 2000; Korniushin & Glaubrecht, 2002) yielded a sphaeriine topology (*Sphaerium* (*Musculium*, *Pisidium*)) in which a sister relationship for *Musculium* and *Pisidium* was supported primarily by a suite of kidney microanatomical characters. In contrast, sphaeriinid gene trees (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002), and one morphological analysis lacking kidney characters (Lee, 2001), recovered a derived clade of asynchronous brooders, e.g. (*Pisidium* (*Musculium*, *Sphaerium*)).

Intrageneric sphaeriinid relationships are poorly defined. A variety of subgeneric groupings have been proposed (see the numerous synonyms listed in Bowden & Heppell, 1968; Clarke, 1973), although few have been widely recognized as valid taxonomic units or explicitly tested phylogenetically (Korniushin & Glaubrecht, 2002). Cooley & Ó Foighil's (2000) preliminary molecular phylogeny recovered paraphyletic *Pisidium* and *Sphaerium* lineages. They, however, refrained from making taxonomic recommendations due to their limited sampling of sphaeriinid diversity and their strictly mitochondrial dataset. The goal of the present study is to construct comprehensive sphaeriinid gene trees, which will provide the basis for an explicit phylogenetically based taxonomy of the group. Sampling effort has been expanded to incorporate sequence data from both nuclear (ITS1 RNA) and mitochondrial (16S RNA) genomes, 38 taxa representing all but one (*Neopisidium*) of the nominal subgeneric sphaeriinid groupings, and samples from multiple continents to test monophyly of nominally cosmopolitan taxa.

MATERIAL AND METHODS

Taxa examined

The 40 sphaeriid taxa examined, their sampling localities, voucher specimen information and GenBank accession numbers are presented in Table 1. We comply with Dreher-Mansur & Meier-Brook's (2000) higher level taxonomic rankings (Family Sphaeriidae, Subfamilies Sphaeriinae and Euperinae), rather than those of Korniushin & Glaubrecht (2002) (Superfamily Pisidioidea, Families Sphaeriidae and Euperidae), because speculation on the superfamily status of this freshwater radiation is premature pending identification of convincing outgroups and the use of superfamily name Pisidioidea violates the ICZN Principle of Coordination (Article 36 ICZN, 1999). Most North American samples were collected by the corresponding author, and the remainder were donated by generous international colleagues. Taxa were chosen to test the monophyly of broadly recognized sphaeriine genera and of subgenera, although we were unable to obtain representatives of the *Pisidium* subgenus *Neopisidium*. While one *Pisidium* species from Ecuador has shell and soft-part anatomic characteristics of the subgenus *Afropisidium* (C. Ituarte pers. comm.), a specific identification has not yet been made. In most cases, at least two individuals were sequenced for each species.

Whenever specimens exhibited considerable variation in shell phenotype, and/or the taxa were collected from different continents, several individuals from multiple localities were sequenced. All different haplotypes obtained were included in the analyses. Two *Eupera* species, *E. cubensis* (Prime, 1865) and *E. platensis* Doello-Jurado, 1921, were selected as outgroups. The sister-group relationship of *Eupera* to the Sphaeriinae has been supported by both morphological (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002) and molecular (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002) studies.

Molecular techniques

Genomic DNA was extracted from either ethanol-preserved or frozen (–70°C) tissue. About 20–30 mg of mantle tissue per individual (or the whole animal in the case of the smaller specimens) was processed with a DNeasy Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Fragments of two different ribosomal genes were amplified for each species. A ~480 nt (nucleotide) fragment of the mitochondrial large ribosomal subunit (16S) was amplified using primers 16Sar and 16Sbr (Kessing *et al*., 1989) for most taxa – a subset ($N = 22$) of 16S sphaeriinid sequences being available from a previous study (Cooley & Ó Foighil, 2000). The entire nuclear ribosomal first internal transcribed spacer (ITS1) was amplified using primers annealing to flanking regions of 18S and 5.8S genes (White, McPheron & Stauffer, 1996). The target fragments were amplified with *Taq* DNA Polymerase (Promega, Madison, WI, Buffer A) and a negative control (no template) was included in each amplification run. For all reactions, a touchdown protocol (Palumbi, 1996) was utilized. An initial annealing temperature of 65°C was decreased by 2°C/cycle until the final annealing temperature (45–50°C for 16S and 50–55°C for ITS1) was reached and subsequently maintained for an additional 35 cycles. The resulting PCR products were isolated on 1% agarose gels, excised over UV light, and purified using a QIAEX II Gel Extraction Kit (Qiagen). Sequencing reactions were performed using BigDye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer Applied Biosystems, Palo Alto, CA) with the respective original PCR primers (annealing temperature 45°C for 16S, 50°C for ITS1) for both strands of amplified products. Excess dyes were removed from sequencing reaction products using Centri-sep spin columns (Princeton Separations, Adelphia, NJ) loaded with G-50 Sephadex (Sigma, St. Louis, MO). Sequencing products were electrophoresed on an ABI 377 automated DNA sequencer.

Sequence alignment and phylogenetic analyses

Sequence chromatograms were edited manually by comparing both strands for all taxa using Sequence Navigator ver. 1.0.1 (Applied Biosystems). They were compiled with Sequence Monkey ver. 2.9.0 (Graf, 2000), aligned using ClustalX (Thompson *et al*., 1997) and the alignment was refined manually where necessary. ITS1 sequences show a high degree of similarity among congeners of *Sphaerium* and *Musculium*, and among *Pisidium* subgenera, but these groupings are quite distinct from each other for this gene fragment. To accommodate this pattern, we first generated 10 independent alignment files corresponding to subgenera (two for *Cyclocalyx* due to pronounced length differences and one for *Sphaerium s.s.* and *Herringtonium*) and these were serially combined into one master alignment using ClustalX. ITS1 sequence variation was concentrated at the 5′ end and was maximal for ingroup/outgroup comparisons. To check whether ambiguous alignment generates any significant phylogenetic conflict, we analysed the complete ITS1 alignment for all taxa, a truncated dataset [in which highly variable sections (positions 44–97nt) were deleted] for all taxa, and also an ingroup only nontruncated dataset. Both 16S and ITS1 alignments can be downloaded from the UMMZ Mollusk division web (http://www.ummz.lsa.umich.edu/mollusks/people/taehwan.html). In order to test if each data set has a hierarchical structure, the degree of skewness (g_1; Hillis & Huelsenbeck, 1992) was calculated (500 000 randomly sampled trees) and permutation tests (1000 replicates) were conducted using PAUP* ver. 4.0b8 (Swofford, 2002).

The 16S and ITS-1 datasets were phylogenetically analysed as either individual or combined (16S + ITS1) matrices using PAUP*. A 32 character morphological matrix (Lee, 2001) was generated for many of the North American taxa studied. However, we did not incorporate this matrix into our combined analyses because many of the character states for the non-North American taxa are presently unknown to us. Maximum parsimony (MP) analyses were performed on each partition including modified ITS-1 datasets and on the combined data. Heuristic searches were conducted using equal character weighting, 100 random stepwise addition and tree bisection-reconnection (TBR) branch-swapping. Inferred sequence gaps were considered as missing data. Branch support levels were estimated with bootstrapping (Felsenstein, 1985) (200 replications, heuristic searches, 10 random additions each) using PAUP*, and also with Bremer decay index values (Bremer, 1994) calculated using TreeRot ver. 2 (Sorenson, 1999), which generates a constraint file for PAUP*. Because of the extensive computational time, the maximum number of trees to

Table 1. Catalogue of the studied taxa, voucher specimen information (UMMZ: University of Michigan, Museum of Zoology; DMNH: Delaware Museum of Natural History), sampling localities and GenBank accession numbers. Species identifications were performed by the respective collectors, unless otherwise indicated (*identified by Kuiper JGJ), and confirmed by the corresponding author

Species	Sample No.	Locality	Collector	Catalogue No.	GenBank accession No.	
					16S	ITS1
Class Bivalvia						
Order Veneroida						
Family Sphaeriidae Deshayes, 1854 (1820)						
Subfamily Euperinae Heard, 1965						
Eupera cubensis (Prime)		Havana, Cuba	M. Yong	UMMZ266709	AY093549	AY093501
E. platensis Doello-Jurado		Buenos Aires, Argentina	C. Ituarte	UMMZ265505	AF152026	AY093502
Subfamily Sphaeriinae Baker, 1927						
Musculium argentinum (d'Orbigny)		Buenos Aires, Argentina	C. Ituarte	UMMZ266668	AF152034	AY093503
M. japonicum (Westerlund)		Ehime Prefecture, Japan	H. Ieyama	UMMZ266720	AY093550	AY093504
M. lacustre (Müller)	(EU)	Tübingen, Germany	C. Meier-Brook	UMMZ266754	AY093551	AY093505
	(NA)	Michigan, USA	T. Lee	UMMZ266755	AY093552	AY093506
M. miyadii Mori*		Kuril Islands, Russia	T. Pearce	DMNH209359	AY093553	AY093507
M. partumeium (Say)		Michigan, USA	T. Lee	UMMZ266670	AF152036	AY093508
M. securis (Prime)		Michigan, USA	T. Lee	UMMZ266667	AF152033	
		Michigan, USA	T. Lee	UMMZ266710		AY093509
M. transversum (Say)		Michigan, USA	R. Mulcrone	UMMZ266722	AY093554	AY093510
Pisidium (*Afropisidium*) *sterkianum* Pilsbry		Buenos Aires, Argentina	C. Ituarte	UMMZ265503	AF152032	AY093512
P. (*A.*) sp.		Río Pastaza, Ecuador	J. Sparks	UMMZ266723	AY093555	AY093511
P. (*Cyclocalyx*) *adamsi* Stimpson	(NA1)	Michigan, USA	T. Lee	UMMZ266716	AY093556	AY093513
	(NA2)	Michigan, USA	T. Lee	UMMZ266663	AF152031	AY093548
P. (*C.*) *casertanum* (Poli)	(AS)*	Kuril Islands, Russia	T. Pearce	DMNH209351		AY093514
	(EU)	Ammerbuch, Germany	C. Meier-Brook	UMMZ266726	AY093557	AY093515
	(NA1)	Michigan, USA	T. Lee	UMMZ266727	AY093558	AY093516
	(NA2)	Michigan, USA	T. Lee	UMMZ266728	AY093559	AY093517
P. (*C.*) *compressum* Prime	(NA1)	Michigan, USA	T. Lee	UMMZ266664	AF152029	
	(NA2)	Michigan, USA	T. Lee	UMMZ266714	AY093560	AY093518
P. (*C.*) *fallax* Sterki		Michigan, USA	T. Lee	UMMZ266730	AY093561	AY093519
P. (*C.*) *hallae* Kuiper		Sydney, Australia	A. Korniushin	UMMZ266731	AY093562	AY093520
P. (*C.*) *hibernicum* Westerlund		Heiliges Meer, Germany	C. Meier-Brook	UMMZ266732	AY093563	AY093522

P. (*C.*) *lilljeborgi* Esmark & Hoyer*		Kuril Islands, Russia	T. Pearce	DMNH210870		AY093521
P. (*C.*) *milium* Held	(EU)	Heiliges Meer, Germany	C. Meier-Brook	UMMZ266733	AY093564	AY093523
	(NA)	Michigan, USA	T. Lee	UMMZ266666	AF152028	AY093524
P. (*C.*) *nipponense* Kuroda		Gunma Prefecture, Japan	H. Ieyama	UMMZ266734	AY093565	AY093525
P. (*C.*) *nitidum* Jenyns		Heiliges Meer, Germany	C. Meier-Brook	UMMZ266735	AY093566	AY093526
P. (*C.*) *personatum* Malm		Ammerbuch, Germany	C. Meier-Brook	UMMZ266737	AY093567	AY093527
P. (*C.*) *subtruncatum* Malm		Heiliges Meer, Germany	C. Meier-Brook	UMMZ266738	AY093568	AY093528
P. (*C.*) *supinum* Schmidt		Hopstener Ach., Germany	C. Meier-Brook	UMMZ266739	AY093569	AY093529
P. (*C.*) *variabile* Prime		Michigan, USA	T. Lee	UMMZ266665	AF152030	AY093530
P. (*Odhneripisidium*) *parvum* Mori		Ehime Prefecture, Japan	H. Ieyama	UMMZ266744	AY093570	AY093531
P. (*O.*) *japonicum* Pilsbry & Hirase		Nagano Prefecture, Japan	H. Ieyama	UMMZ266743	AY093571	AY093532
P. (*Pisidium*) *amnicum* (Müller)		Goian, Pontevedra, Spain	R. Araujo	UMMZ266745	AY093572	
P. (*P.*) *dubium* (Say)		Michigan, USA	T. Lee	UMMZ265502	AF152027	
		Michigan, USA	T. Lee	UMMZ266715		AY093533
Sphaerium (*Sphaerium*) *baicalense* Dybowski		Bolshye Koty, Russia	A. Korniushin	UMMZ266759		AY093534
S. (*S.*) *corneum* (Linnaeus)	(EU1)	Germany	O. Heilmayer	UMMZ265501	AF152037	AY093535
	(EU2)	Rennes, France	M. Siddall	UMMZ255296		AY093547
S. (*S.*) *fabale* (Prime)		Michigan, USA	T. Lee	UMMZ266673	AF152039	
		Michigan, USA	T. Lee	UMMZ266747		AY093536
S. (*S.*) *nucleus* (Studer)		Ukraine	A. Korniushin	UMMZ266761	AY093573	AY093537
S. (*S.*) *rhomboideum* (Say)		Michigan, USA	T. Lee	UMMZ266672	AF152038	
		Michigan, USA	T. Lee	UMMZ266762		AY093538
S. (*S.*) *rivicola* (Lamarck)		Germany	O. Heilmayer	UMMZ266749		AY093539
S. (*S.*) *simile* (Say)		Michigan, USA	T. Lee	UMMZ266712	AF152040	AY093540
S. (*S.*) *striatinum* (Lamarck)	(NA1)	Michigan, USA	L. Cooley	UMMZ266675	AF152041	
	(NA2)	Michigan, USA	T. Lee	UMMZ266677	AF152042	AY093541
	(NA3)	Michigan, USA	T. Lee	UMMZ266678	AF152043	AY093545
	(NA4)	Michigan, USA	T. Lee	UMMZ266679	AF152044	AY093546
	(NA5)	Michigan, USA	L. Cooley	UMMZ266676	AF152045	
S. (*Herringtonium*) *occidentale* (Prime)		Michigan, USA	L. Cooley	UMMZ266671	AF152046	
		Michigan, USA	T. Lee	UMMZ266752		AY093542
S. (*Sphaerinova*) *novaezelandia* Deshayes		New Zealand	S. Roa	UMMZ266680	AF152047	AY093543
S. (*Sph.*) *tasmanicum* (Tenison Woods)		NSW, Australia	A. Korniushin	UMMZ266681	AF152048	AY093544

be saved was limited to 5000 when calculating bootstrap and decay index values for the ITS1 data set. Alternative MP topologies were explored using MacClade ver. 3.07 (Maddison & Maddison, 1992). A maximum likelihood (ML) analysis was also performed on the combined matrix under the HKY model (Hasegawa, Kishino & Yano, 1985). The transition/transversion ratio and base frequencies were estimated from the data using the single MP tree obtained. The estimated parameters were then used in ML searches (heuristic searches, five random stepwise addition, TBR branch-swapping). Bootstrap estimates for ML topology were assessed with 100 replicates using the 'fast' stepwise-addition option for heuristic searches. For both MP and ML analyses, two *Eupera* species, *E. cubensis* and *E. platensis*, were designated as outgroups and sphaeriinid taxa were forced to be monophyletic and sister to monophyletic outgroups in order to root the phylogeny.

RESULTS

We were largely successful in our attempt to characterize 40 sphaeriid taxa, including two *Eupera* species, for both ribosomal gene fragments. Thirty-seven taxa were tractable for the mt gene, the exceptions being *Pisidium lilljeborgi* Esmark & Hoyer, 1886, *Sphaerium baicalense* Dybowski, 1902 and *S. rivicola* (Lamarck, 1818), and a total of 47 16S genotypes (incorporating 21 from Cooley & Ó Foighil, 2000) were available for phylogenetic analyses (Table 1). Supplemental individuals of a number of Cooley & Ó Foighil's (2000) study taxa were sequenced, including European specimens. In doing so, we discovered an error in the previous study: transposition of the identities of the *Pisidium adamsi* Stimpson, 1851 and *P. milium* Held, 1836 sequences. Their respective GenBank submissions have been corrected. All but one taxon, *Pisidium amnicum* (Müller, 1774), were successfully characterized for the nuclear gene fragment and a total of 48 ITS1 genotypes were recovered.

Including inferred gaps, the ribosomal 16S data set contained 480 aligned sites and, of these, 144 characters were parsimony-informative. Considerable sequence length variation was observed in the ITS1 data set. Although most ITS1 genotypes ranged in length from 504 to 551 nt, significantly longer sequences, ranging from 655 (*Pisidium adamsi*) to 682 nt (*P. supinum* Schmidt, 1850), were obtained from a majority of the subgenus *Cyclocalyx* investigated. Alignment of the ITS1 sequences using ClustalX resulted in a matrix of 760 aligned sites and all of the longer genotypes are inferred to have an ~160 nt long insertion at the same position. Although intraindividual ITS1 heterozygosity has been reported from some insects, crustaceans and trematodes (Wesson, Porter & Collins, 1992; Vogler & DeSalle, 1994; van Herwerden, Blair & Agatsuma, 1999; Harris & Crandall, 2000), our directly sequenced sphaeriid ITS1 sequences did not display observable signs of intraindividual heterozygosity expected from either nucleotide substitution or from insertion/deletion events. A significant hierarchical structure existed in the molecular data sets according to PTP ($P = 0.001$ for both data sets) and degrees of skewness (16S $g_1 = -0.396$; ITS1 $g_1 = -0.694$) tests.

Four equally most-parsimonious trees of 526 steps (CI = 0.447; RI = 742) were obtained from the analysis of mt 16S dataset and a strict consensus was recovered (Fig. 1). Although the parsimony analysis of nuclear ITS1 dataset yielded numerous trees (No. of trees = 1040; L = 445; CI = 0.724; RI = 0.886), most polytomies were restricted to tip clades and the recovered strict consensus was well resolved (Fig. 2). Unrooted analysis of ingroup ITS1 recovered the same topology, and the analysis of a truncated ITS1 dataset (without highly variable positions 44–97nt) resulted in a largely congruent tree topology to that of complete dataset: the only difference was losing resolution among the *Sphaerium occidentale* (Prime, 1856) and *S. rhomboideum* (Say, 1822) clade, the *S. corneum* (Linnaeus, 1758) *S. baicalense* and *S. nucleus* (Studer, 1820) clade, and the *Sphaerinova* clade.

The trees depicted in Figures 1 and 2 differ in a number of topological details that reflect distinct, and largely complementary, tempos of molecular substitution for the 16S and ITS1 target gene fragments. Pairwise comparisons of the study taxa clearly show that molecular substitutions (for the gene fragments assayed) accrue more rapidly in sphaeriid 16S than in their ITS1 gene fragments. Although both gene fragments contain phylogenetic information throughout sphaeriid treespace, in general, 16S data generated enhanced resolution in tip clades, whereas ITS1 sequences better preserved stem (plesiomorphic) resolution (Figs 1, 2). Parsimony analysis of the combined (16S + ITS1) dataset yielded a single most-parsimonious tree of 951 steps (CI = 0.568; RI = 0.793; Fig. 3), although a polytomous relationship was recovered for European and North American *Musculium lacustre* (Müller, 1774) genotypes, together with the branch supporting the *M. japonicum* (Westerlund, 1883) and *M. miyadii* Mori, 1933 clade, due to a complete lack of synapomorphy. ML analysis of the combined matrix recovered a largely congruent topology (Ln likelihood = −7034.61154) to the MP tree: the only difference was the position of *Pisidium dubium* (Say, 1816) being sister to *Sphaerium*/*Musculium* clade in the ML tree. Phylogenetic placement of *P. dubium*, however, was not supported by either MP or ML bootstrapping (Fig. 3). A number of major topological features were common to all three gene trees (Figs 1–3) including the presence of an exclusive clade of asynchronously

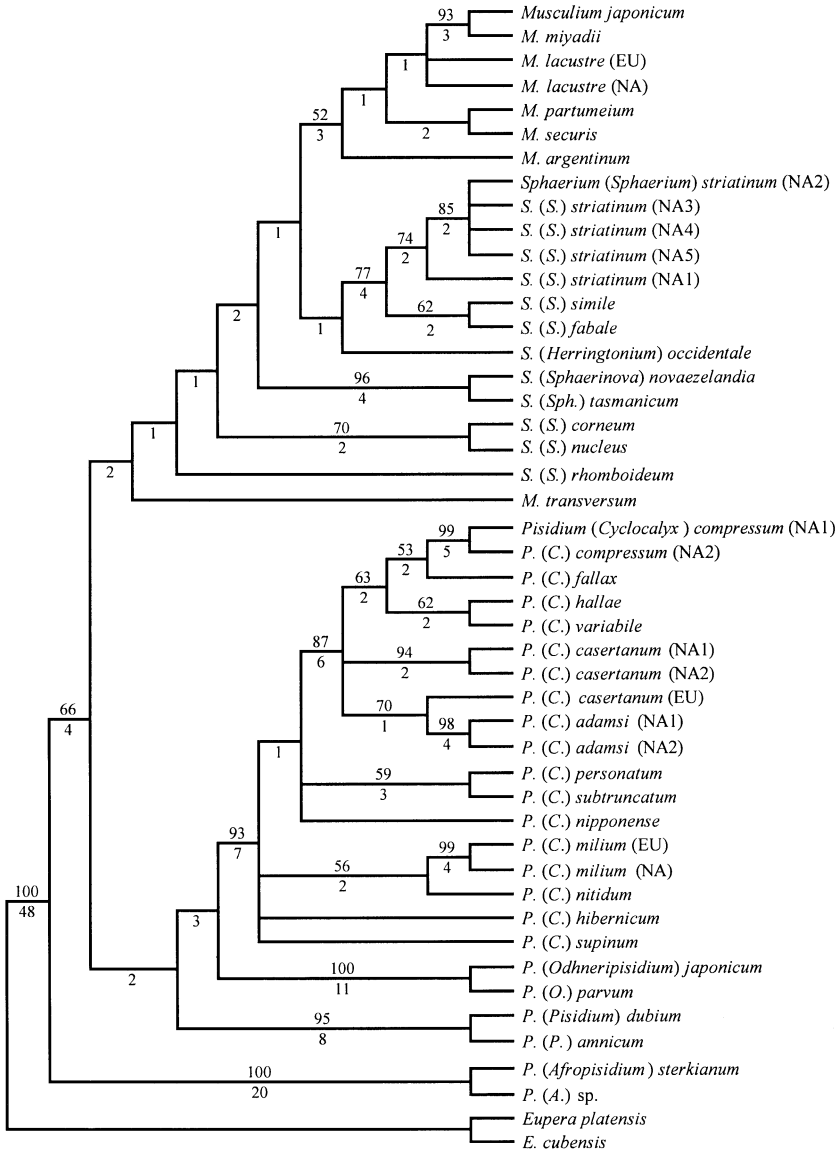

Figure 1. Strict consensus of the four equally most parsimonious trees (L = 526; CI = 0.447; RI = 0.743) obtained from the phylogenetic analysis of sphaeriid mitochondrial 16S rDNA sequences. Two *Eupera* species, *E. cubensis* and *E. platensis*, were designated as outgroups and inferred sequence gaps were considered as missing data. Numbers above the branches represent bootstrap values and numbers below indicate decay index values.

brooding *Sphaerium* and *Musculium* taxa nested among a paraphyletic grade of synchronously brooding *Pisidium* lineages.

Asynchronous brooders

Support levels for *Sphaerium*/*Musculium* monophyly, and for most internal nodes in this clade of asynchronous brooders, were most pronounced in the combined MP and ML analyses (Fig. 3) which yielded five robustly supported terminal clades. Neither *Musculium* nor *Sphaerium* were monophyletic. One of the terminal clades found in Figures 1–3 encompassed six of the seven *Musculium* taxa studied, including the type species, *M. lacustre*, and both Holarctic and South American representatives. The exception was the North American taxon *M. transversum* (Say, 1829), which was basal to the remaining asynchronous brooders in the 16S tree (Fig. 1), but sister to a terminal clade of North American *Sphaerium s.s.* species (+ the European *S. rivicola* in Fig. 2) when the ITS1 data was considered (Figs 2, 3). Three other well-supported terminal *Sphaerium* clades were recovered in the combined analyses (Fig. 3), including an antipodean

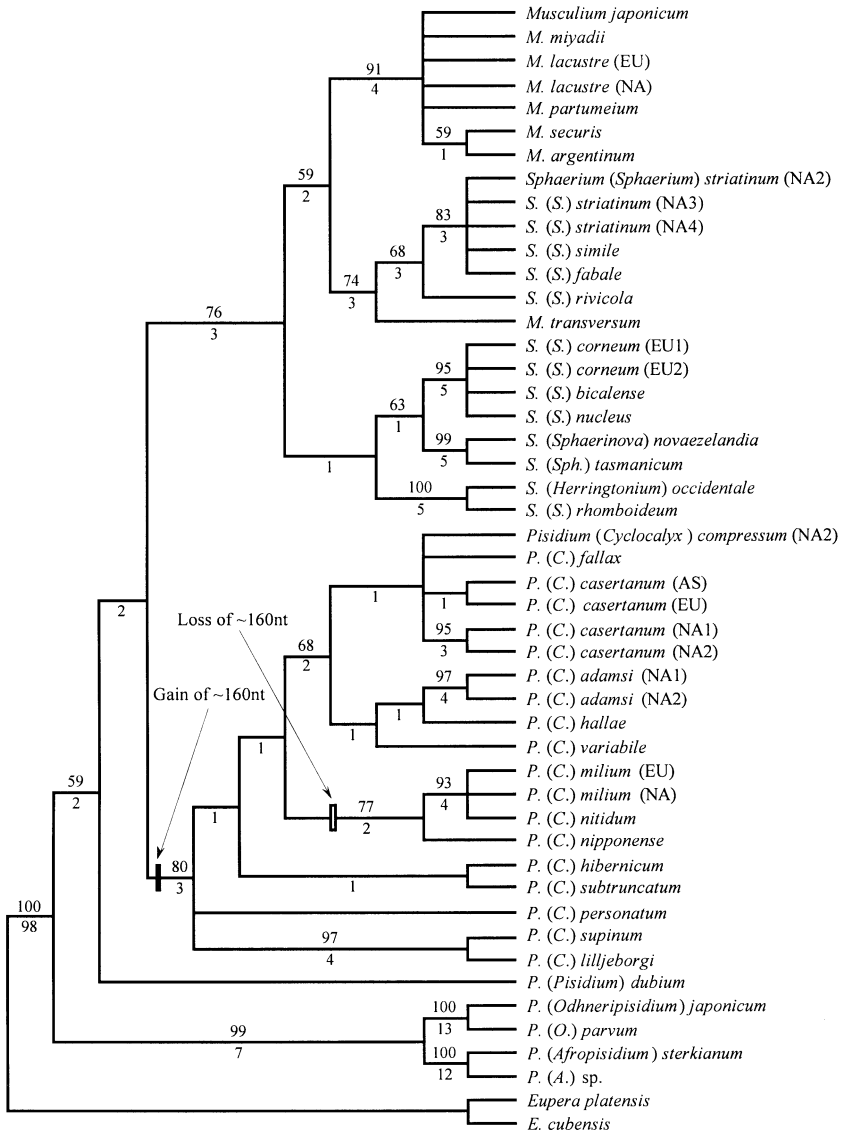

Figure 2. Strict consensus of the 1040 equally most parsimonious trees (L = 445; CI = 0.724; RI = 0.886) obtained from the phylogenetic analysis of sphaeriid nuclear ITS1 rDNA sequences. The inferred evolutionary gain and loss of a ~160 nt fragment are indicated. Two *Eupera* species, *E. cubensis* and *E. platensis*, were designated as outgroups and inferred sequence gaps were considered as missing data. Numbers above the branches represent bootstrap values and numbers below indicate decay index values.

Sphaerinova clade and a Eurasian *Sphaerium corneum* clade. Rounding out the asynchronous brooders, the ITS1 data support a robust sister relationship for *S. rhomboideum* and *S. occidentale* (the only species of the monotypic subgenus *Herringtonium*) which were placed basally in the combined analyses (Fig. 3).

Synchronous brooders

The genus *Pisidium* produced paraphyletic topologies in all our analyses (Figs 1–3). Each topology contained four robustly supported clades, representing the four *Pisidium* subgenera sampled (*Afropisidium*, *Odhneripisidium*, *Pisidium s.s.*, and *Cyclocalyx*), whose relative branching order changed among 16S and ITS1 datasets as well as among combined MP and ML topologies. In all MP analyses, the most deeply nested of the four *Pisidium* subgeneric clades consisted of cosmopolitan members of the subgenus *Cyclocalyx* which, in the ITS1-containing analyses (Figs 2, 3), was sister to the asynchronous-brooding *Sphaerium/Musculium* clade. Intriguingly, the *Cyclocalyx* clade, whose monophyly was robustly supported in all analyses, is distinguished by a molecular synapomorphy. A ~160 nt

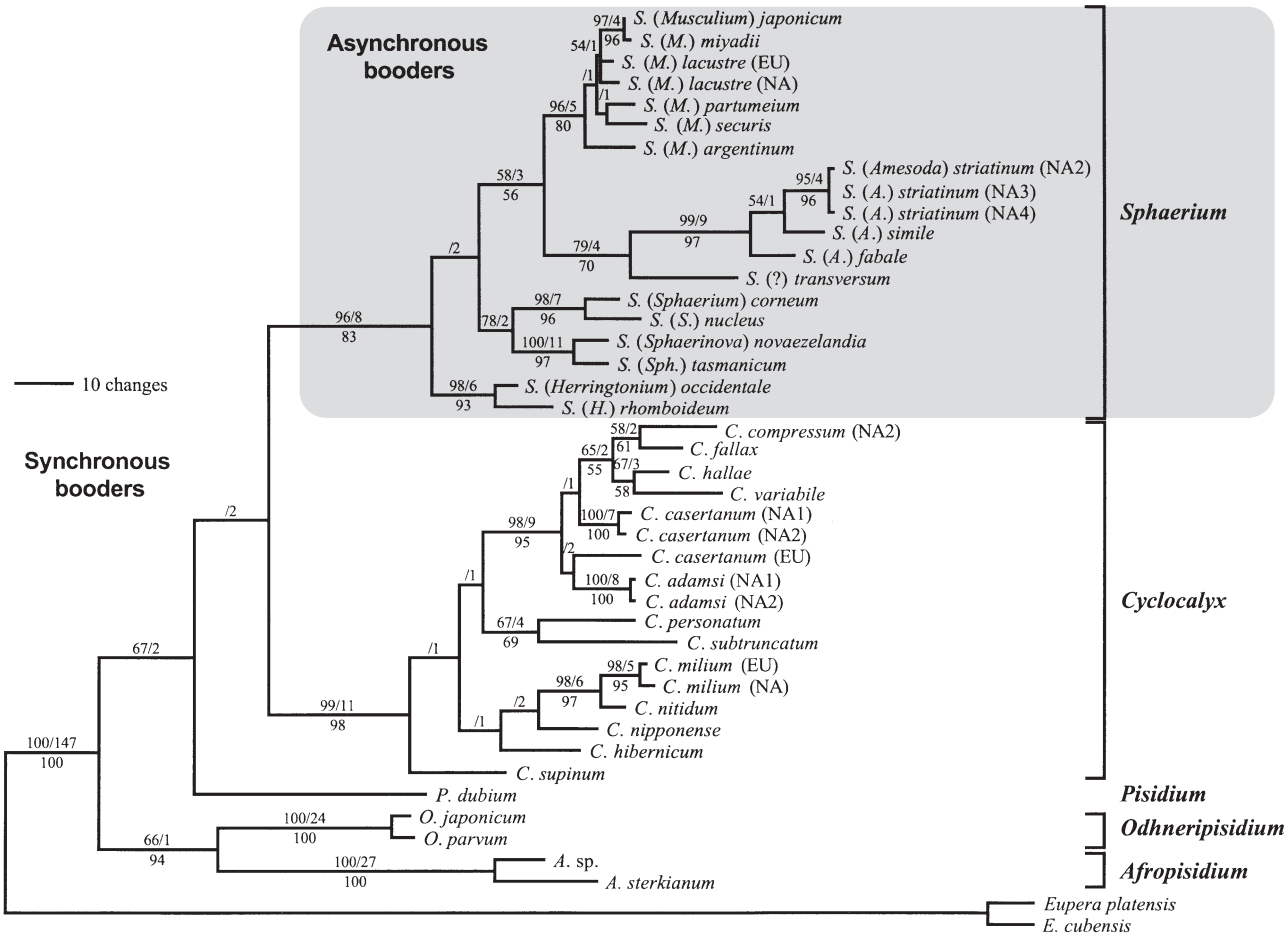

Figure 3. The single most-parsimonious tree (L = 951; CI = 0.568; RI = 0.793) obtained from the maximum parsimony analysis of combined (16S + ITS1) sequence dataset. Maximum likelihood analysis produced a largely congruent topology (HKY model; Ln likelihood = −7034.61154) with the only difference being *Pisidium dubium* sister to *Sphaerium/Musculium* clade. Taxonomic names are arranged according to suggested sphaeriinid taxonomy in the present study and five major monophyletic lineages are indicated. Two *Eupera* species, *E. cubensis* and *E. platensis*, were designated as outgroups. MP bootstrap values are shown to the left of the slash and decay index values to the right above the branches. Numbers below the branches indicate ML bootstrap values.

insertion in the ITS1 fragment appears on the gene tree topologies along the stem branch of the *Cyclocalyx* clade and is secondarily lost in a tip clade containing *P.* (*C.*) *milium, P.* (*C.*) *nitidum* Jenyns, 1832 and *P.* (*C.*) *nipponense* Kuroda, 1928 (Fig. 2). An unanticipated feature of the gene trees is the presence of a robust internal clade among the *Cyclocalyx* taxa which incorporates *P.* (*C.*) *compressum* Prime, 1852, *P.* (*C.*) *fallax* Sterki, 1896, *P.* (*C.*) *hallae* Kuiper, 1983, *P.* (*C.*) *variabile* Prime, 1852, *P.* (*C.*) *casertanum* and *P.* (*C.*) *adamsi* (Figs 1–3). In every analysis, genotypes of the cosmopolitan taxon *P. casertanum* were not monophyletic: a European 16S haplotype grouped with *P. adamsi* haplotypes (Fig. 1) and European/Asian ITS1 sequences were separated from monophyletic North American haplotypes (Fig. 2).

Each of the remaining three *Pisidium* subgenera studied (*Afropisidium*, *Odhneripisidium*, *Pisidium s.s.*) forms a remarkably well-supported clade, although their relative branching order is unstable. A feature common to all the gene trees is that the *Afropisidium* clade is sister to the remainder of the Sphaeriinae, either alone (16S data) or in conjunction with the *Odhneripisidium* taxa (ITS1-containing datasets). The *Odhneripisidium* species are sister to the *Cyclocalyx* clade when 16S alone is considered (Fig. 1). The *Pisidium s.s.* clade, represented by *P. dubium*, together with the type species *P. amnicum* in the 16S alone, is nested in an intermediate position, sister either to the (*Cyclocalyx* (*Sphaerium*, *Musculium*)) clade in MP or to the *Sphaerium/Musculium* clade in ML analysis of the combined data.

DISCUSSION

CONGRUENCE WITH PREVIOUS SPHAERIID CLADISTIC STUDIES

Comparison of our results with those obtained in recent molecular (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002) and morphological (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002) cladistic analyses of the Sphaeriidae reveals some intriguing points of agreement, in addition to substantial areas of incongruence.

Although they differ in some topological details, there is a striking level of congruence among all of the sphaeriid gene trees generated in this, and in previous (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002) studies, for major elements of tree structure, i.e. *Pisidium* paraphyly and a derived clade of asynchronous brooders. This is a notable result, given the wide range of taxonomic sampling intensities (maximal in the present study) and the diversity of the genes employed: nuclear ribosomal RNA [28S (Park & Ó Foighil, 2000), ITS1 (present study)], nuclear single copy 6-phosphogluconate dehydrogenase (Lee & Ó Foighil, 2002) and mitochondrial 16S ribosomal RNA (Cooley & Ó Foighil, 2000; present study).

There is less topological congruence among the molecular and morphological analyses, and indeed among the individual morphological studies (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002). The most clear-cut distinction involves *Pisidium* taxa which are recovered as monophyletic in the morphological trees and paraphyletic in the gene trees. This discrepancy is not particularly surprising because a relative lack of synapomorphies defining ingroup phylogenetic relationships is a weakness common to all three sphaeriid morphological matrices (Dreher-Mansur & Meier-Brook, 2000; Lee, 2001; Korniushin & Glaubrecht, 2002). The *Pisidium* molecular datasets are more character-rich than their morphological counterparts and therefore have an enhanced scope to reveal within-*Pisidium* phylogenetic structure. Nevertheless, gene tree sister relationships among the four robust terminal clades, representing the *Pisidium* subgenera sampled, are tentative with the apparent exception of the strongly supported *Odhneripisidium*/*Afropisidium* clade evident in the ITS1 tree (Fig. 2).

The most pointed element of incongruence among the molecular and a subset of the morphological analyses concerns the topological placement of the asynchronous brooding taxa: *Sphaerium* and *Musculium*. Previous molecular studies, together with one of the morphological analyses (Lee, 2001), recovered a derived monophyletic clade of asynchronous brooders (*Eupera* (*Pisidium* (*Musculium*, *Sphaerium*))) and this result is unambiguously confirmed by our new data (Fig. 3). In contrast, Dreher-Mansur & Meier-Brook's (2000) and Korniushin & Glaubrecht's (2002) cladistic analyses recovered a (*Eupera* (*Sphaerium* (*Musculium*, *Pisidium*))) topology.

Sphaeriid morphological datasets have therefore generated two distinct topologies for the asynchronous brooders and this reflects qualitative differences in the character sets chosen and in their outgroup rooting. Lee (2001) restricted his character set to major anatomical and developmental features that have been considered fundamental by previous workers and, being aware of the potential for significant convergent evolution in brooding character states in corbiculid outgroup taxa (Dreher-Mansur & Meier-Brook, 2000; Park & Ó Foighil, 2000), analysed ingroup characters without rooting in addition to outgroup rooted analysis. Conversely, Dreher-Mansur & Meier-Brook (2000) and Korniushin & Glaubrecht (2002) incorporated a number of fine-scale anatomical features, especially numerous, potentially nonindependent, details of kidney substructure [$N = 17$, Dreher-Mansur & Meier-Brook (2000); $N = 12$, Korniushin & Glaubrecht (2002)], in their datasets and did not test for outgroup rooting problems. These methodological distinctions underlay the differential topological results, and the inferred supporting synapomorphies, generated by the their studies. For instance, Lee's (2001) *Sphaerium*/*Musculium* clade (bootstrap value = 80) is diagnosed with one unambiguous (short presiphonal suture) and two ambiguous (asynchronous brooding, nonpartitioned brood sac) synapomorphies. When corbiculid outgroups are excluded from the analysis, all three characters, plus an additional one (fused siphons), unambiguously diagnose the *Sphaerium*/*Musculium* clade (bootstrap value = 95) within the Sphaeriidae (Lee, 2001). Dreher-Mansur & Meier-Brook's (2000) *Pisidium*/*Musculium* clade is supported by three kidney and one shell character (no support values given) and that of Korniushin & Glaubrecht (2002) (bootstrap support <50) by one of these three kidney characters. The (*Sphaerium* (*Musculium*, *Pisidium*)) topology (total of 154 steps, 752 most parsimonious trees) in the latter study is far from robust given that the alternative topology (*Pisidium* (*Musculium*, *Sphaerium*)) is a mere one step longer (Korniushin & Glaubrecht, 2002). In contrast, producing the (*Sphaerium* (*Musculium*, *Pisidium*)) topology requires an extra two steps in the Lee (2001) morphological dataset and at least 22 additional steps in our combined 16S and ITS1 dataset.

Although there is a dichotomy of opinion in the recent morphologically based cladistic literature concerning the interrelationships of the asynchronous brooding taxa (*Sphaerium* and *Musculium*), it is now

clear that the weight of evidence supports their designation as a derived clade within the Sphaeriinae. This conclusion stems not only from our new results (Fig. 3), but is a robustly consistent feature of independent phylogenetic tree topologies based on four diverse gene fragments (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002; present study) and on a conservatively chosen suite of morphological characters (Lee, 2001). It is also consistent with earlier taxonomic studies, many of which centred on the vexed question of whether or not *Sphaerium* and *Musculium* taxa were sufficiently distinct to warrant separate generic status (Sterki, 1909; Ellis, 1962; Herrington, 1962; Gale, 1972; Clarke, 1973; Mackie & Qadri, 1974; Heard, 1977; Hornbach, McLeod & Guttaman, 1980).

The phylogenetic placement of the asynchronous brooders is important because it shapes our view of the primary evolutionary trends within the sphaeriinid clade. For instance, a number of workers have identified a series of conspicuous character reductions in smaller sized *Pisidium* taxa, proposed that miniaturization represents the predominant evolutionary trend in the Sphaeriinae, and concluded that the larger species of *Sphaerium* represent the plesiomorphic condition in this subfamily (Meier-Brook, 1970, 1977; Korniushin, 1998a,b; Dreher-Mansur & Meier-Brook, 2000; Korniushin & Glaubrecht, 2002). Conversely, our data consistently place *Sphaerium* in a derived sphaeriinid clade of asynchronous brooders, revealing that large-bodied *Sphaerium* taxa, and asynchronous brooding, are derived states in this subfamily.

Taxonomic implications

Based on their phylogenetic tree topologies, Korniushin & Glaubrecht (2002) proposed a comprehensive taxonomic revision involving the erection of 10 sphaeriinid genera. Although large areas of their treespace have very poor support levels, and are incongruent with our results (Fig. 3), there are also some intriguing areas of topological congruence among the morphological and molecular datasets. Previous molecular phylogenetic studies of the Sphaeriidae did not make taxonomic recommendations due primarily to insufficient sampling of global sphaeriid diversity (Cooley & Ó Foighil, 2000, Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002). With the notable absence of *Neopisidium* representatives, the taxon sampling of our present study is quite comprehensive and contains a number of robustly supported clades that have significant taxonomic import.

Our results indicate that the present taxonomy of the Sphaeriinae needs, in large part, to be revised, and new generic and subgeneric classifications that are strongly supported by our new data are summarized in Figure 3. Instead of the traditional three cosmopolitan sphaeriine genera, five well-defined and robustly supported monophyletic lineages are here recognized as generic level groups (four were traditionally assigned to *Pisidium* subgenera) although the generic status of two (*Afropisidium* and *Odhneripisidium*) needs further verification.

Pisidium s.s. Pfeiffer, 1821 (Type species: *Tellina amnica* Müller, 1774)

This lineage is represented by the North American *P. dubium* and, in the 16S dataset, the European *P. amnicum*. *Pisidium s.s.* is one of the least diverse of the sphaeriinid genera, nominally including just one additional species (*P. idahoense*), and its members are characterized by their relatively large and thick shells among the traditional *Pisidium* taxa and possession of two complete demibranchs (Heard, 1966). Although its monophyly is well-supported, both in our mt gene tree (Fig. 1: BS = 95, DI = 8) and in Korniushin & Glaubrecht's (2002) cladograms (BS = 82), the topological placement of *Pisidium s.s.* relative to the other synchronously brooding sphaeriinids is poorly defined at present.

Cyclocalyx Dall, 1903 (Type species: *Cyclas obtusalis* Lamarck, 1818)

Cyclocalyx taxa (see the nomenclatural discussion in Clarke, 1973: 168) are of intermediate size among the traditional *Pisidium* taxa, possess 1.5 demibranchs (Heard, 1966), and were represented in our dataset by 13 species sampled from North America, Europe, eastern Asia, and Australia. All of our *Cyclocalyx* taxa were robustly monophyletic and, being sister to the asynchronous brooders in ITS1-containing analyses (Figs 2, 3), consistently formed the most derived clade of synchronous-brooding Sphaeriinae in every MP topology. Parsimony analysis of the ITS1 fragment revealed a generic-level molecular synapomorphy in the form of a ~160 nt insertion that predated radiation of the genus and was secondarily lost in the stem lineage of the (*C. nipponense* (*C. milium*, *C. nitidum*)) tip clade (Fig. 2). Alternative hypotheses, a single origin without secondary loss or multiple origins of the insertion, require either more steps (six in ITS1 data alone or 20 in combined data) or independent attainment of the condition in each species. Basal lineages within the *Cyclocalyx* clade were primarily composed of Old World taxa, and most of the New World lineages studied were present in a well-supported (Fig. 3: MPBS = 98, DI = 9, MLBS = 95) terminal clade, including the

cosmopolitan *C. casertanum* and Australian *C. hallae*. Northern Hemisphere samples of *C. casertanum* were paraphyletic for both nuclear and mitochondrial genotypes, indicating that this morphologically variable cosmopolitan taxon incorporates a complex of cryptic species.

With a few exceptions, it is difficult to meaningfully compare our *Cyclocalyx* results with those of Korniushin & Glaubrecht (2002) because the taxa sampled, topologies generated and systematic conclusions reached are quite different. Where we get a robustly supported derived clade (Fig. 3: MPBS = 99, DI = 11, MLBS = 98), the morphological study generated a weakly supported (BS ≤ 64) paraphyletic grade of lineages nested centrally within the synchronous-brooding sphaeriinids. Korniushin & Glaubrecht (2002) recognized four terminal clades which they raise to generic status (*Henslowiana*, *Casertiana*, *Cingulipisidium* and *Cyclocalyx*) although all four are far from robust (BS < 50). A mixed picture of congruence emerges for the limited sets of replicate taxa in the two studies. *C. lilljeborgi* and *C. supinum*, placed in *Henslowiana* by Korniushin & Glaubrecht (2002), are convincing sister taxa in the ITS1 gene tree (Fig. 2) and traces of the robust terminal clade of primarily North American *Cyclocalyx* taxa (Figs 1–3) are seen in the placement of *C. compressum* and *C. casertanum* in their *Casertiana* clade. However, there are some notable discrepancies, e.g. our data solidly support a *C. nitidum*/*C. milium* sister status (Figs 1–3), although they are placed in separate genera (respectively *Cingulipisidium*/*Cyclocalyx*) in the morphological study.

Such inconsistencies, together with the fragility of the four terminal clades in the morphological study (Korniushin & Glaubrecht, 2002), lead us to conclude that at present these taxa are best placed in a single genus *Cyclocalyx*. There clearly is scope for further taxonomic partitioning, e.g. the robust tip clade containing *C. compressum, C. fallax, C. hallae, C. variabile, C. casertaneum* and *C. adamsi* is a prime candidate for subgeneric status pending the discovery of a morphological synapomorphy. Future taxonomic revision of *Cyclocalyx* will hopefully be based on a synapomorphy-rich data set (including molecular characters) that comprehensively samples this diverse cosmopolitan clade.

Afropisidium Kuiper, 1962 (Type species: *Pisidium pirothi* Jickeli, 1881) and *Odhneripisidium* Kuiper, 1962 (Type species: *Pisidium stewarti* Preston, 1909)

Kuiper (1962) distinguished three *Pisidium* subgenera (*Neopisidium*, *Afropisidium* and *Odhneripisidium*) primarily on the basis of hinge ligament characters. Our sampling of these lineages is suboptimal in that we lack any *Neopisidium* taxa and have a mere two species each of *Afropisidium* (South American *A. sterkianum* Pilsbry, 1897 and an unidentified congener) and *Odhneripisidium* (Asian *O. japonicum* Pilsbry & Hirase, 1908 and *O. parvum* Mori, 1938). Nevertheless, our representatives formed impressively robust (Fig. 3; MPBS = 100 and 100; DI = 24 and 27; MLBS = 100 and 100, respectively) monophyletic *Afropisidium* and *Odhneripisidium* clades, confirming the phylogenetic validity of these taxonomic distinctions. Other compelling aspects of the *Afropisidium*/*Odhneripisidium* clades include their exceptional phylogenetic definition in both mt and nuclear gene trees, and their topological placement. The *Afropisidium* clade was consistently positioned basally within the sphaeriinid clade (Figs 1–3) and this result is congruent with previous molecular studies (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000). Although the molecular data are not unanimous concerning placement of the *Odhneripisidium* clade, the ITS1 data robustly (BS = 99, DI = 7) support a sister relationship to *Afropisidium*. This represents an intriguing note of agreement with the morphological study (Korniushin & Glaubrecht, 2002) which also retrieved a well-supported (BS = 93) clade of *Afropisidium* and *Odhneripisidium* lineages, although, unlike the molecular trees, it is in a derived position among sphaeriinid lineages.

Korniushin & Glaubrecht (2002) included one *Neopisidium* taxon which was weakly (BS = 65) sister to their robust *Afropisidium*/*Odhneripisidium* clade. They proposed amalgamation of all three taxonomic units into a single genus *Neopisidium s.l.*, although the support level of this generic stem branch is much less than that of the next internal node supporting their *Afropisidium*/*Odhneripisidium* clade. Clearly, much more work needs to be done to fully flesh out the evolutionary history of the *Neopisidium*, *Afropisidium* and *Odhneripisidium* lineages.

Sphaerium Scopoli, 1777 (Type species: *Tellina cornea* Linnaeus, 1758)

All of our gene trees exhibit solid support for a derived monophyletic grouping of sequential brooders, *Sphaerium s.l.* and *Musculium,* represented by 15 species sampled from North and South America, Europe, eastern Asia and Australia (Figs 1–3). The data additionally indicate that, as traditionally constructed, *Musculium* and *Sphaerium s.l.* are not natural groups, and we have therefore formed a new classification system for this clade, comprising five subgeneric groupings which are convincingly supported by the available data and are discussed in turn below.

Subgenus *Sphaerium s.s.* Scopoli, 1777

This Eurasian subgenus is represented in our dataset by *S. corneum*, *S. nucleus*, and (ITS1 data only) *S. baicalense*. They formed a well-supported tip clade

that nested among the asynchronous brooders and appeared [in the ITS1-containing datasets (Figs 2, 3)] to be a candidate sister clade to *Sphaerinova* taxa. With one notable exception (*S. rhomboideum*, see *Herringtonium* section below) our *Sphaerium s.s.* results are in general agreement, for replicated taxa, with Korniushin & Glaubrecht's (2002) findings: they place *S. corneum* and *S. nucleus* within a weakly supported (BS < 50) terminal *Sphaerium s.s.* clade. According to Korniushin (1998c), Russian school taxonomists have divided various morphological forms of *S. corneum*, recognized as subspecies or ecophenotypic variations by Western workers, into at least three different genera. Our preliminary data does not raise the expectation that such an elaborate level of taxonomic heterogeneity will turn out to be supportable, e.g. samples from replicate Western European populations of *S. corneum* and from a Lake Baikal congener *S. baicalense* shared an identical ITS1 sequence.

Subgenus *Musculium* Link, 1807 (Type species: *Tellina lacustris* Müller, 1774)

We recovered an exclusive cluster of *Musculium* taxa (sampled from Europe, Asia, North America and South America and including the type species *M. lacustre*) which formed a well-supported derived tip clade among the asynchronous brooders in all our trees, sister to an *Amesoda* clade (Figs 1–3).

The North American *Musculium transversum* was a prominent exception. It was positioned on the stem branch of the *Amesoda* clade in the ITS1-containing datasets (Figs 2, 3), was sister to all the other asynchronous brooders in the 16S trees (Fig. 1), and was part of a paraphyletic grade of *Musculium* and *Sphaerium* taxa in Korniushin & Glaubrecht (2002). Although there is no consensus as to *M. transversum*'s phylogenetic placement, it is clearly not supportable as a *Musculium* taxon and may require a monotypic subgeneric status within *Sphaerium*. This is not a particularly surprising result because a variety of morphological and ecological features are known to distinguish *M. transversum* from the other members of *Musculium*, including larger and thicker shells, the frequent occurrence of noncalyculated beaks, and a preference for riverine over ephemeral habitats (Herrington, 1962; Gale, 1972). Conversely, our results indicate that the ability of *Musculium* clade taxa to effectively colonize ephemeral habitats (Heard, 1977) reflects shared history rather than convergent adaptation and that latent physiological and behavioural synapomorphies may well underlay this facility.

The systematic validity of the cosmopolitan genus *Musculium* has long been controversial among sphaeriid systematists (Sterki, 1909; Ellis, 1962; Herrington, 1962; Gale, 1972; Clarke, 1973; Mackie & Qadri, 1974; Heard, 1977; Hornbach, McLeod & Guttaman, 1980). Our data clearly show that there is a well-supported globally distributed clade of *Musculium* lineages, but that they represent a subgrouping of *Sphaerium* diversity. Retaining generic status for *Musculium* would render *Sphaerium* paraphyletic and would entail assignation of generic names to the five tip clades of asynchronous brooders, which collectively occupy an equivalent area of phylogenetic treespace to the genus *Cyclocalyx* (Fig. 3). We favour retention of the historic *Sphaerium* generic designation and the demotion of *Musculium* to subgeneric status, a taxonomic status that has been previously suggested by a number of workers (Ellis, 1962; Herrington, 1962; Bowden & Heppell, 1968; Clarke, 1973).

Comparing our *Musculium* results for replicated taxa to those of Korniushin & Glaubrecht (2002) reveals points of congruence [sister status of *M. lacustre* and *M. securis* (Prime, 1852), and redesignation of *M. transversum*] and incongruence [positioning of *M. argentinum* (d'Orbigny, 1835)]. These workers place the South American *M. argentinum*, together with Indian and South African *Musculium* taxa, in a hypothesized Gondwanan *Sphaerinova* genus also containing the antipodean *S. tasmanicum* (Tenison Woods, 1876) and *S. novaezelandia* Deshayes, 1854. Their rationale for doing so is not apparent as these taxa form a four-branched polytomy in their shortest phylogenetic trees, thereby providing no evidence to support the existence of a Gondwanan clade. Our data for both nuclear and mt genes unambiguously place *M. argentinum* in a well-supported, predominantly Holartic *Musculium* clade, and moving it to the phylogenetically distinct *Sphaerinova* clade (containing *S. tasmanicum* and *S. novaezelandia*) entails adding 18 steps to the combined data (Fig. 3) MP treelength.

Subgenus *Amesoda* Rafinesque, 1820 (Type species: *Cyclas similis* Say, 1816)

The North American *Amesoda* (see the nomenclatural discussion in Clarke, 1973: 135) clade [*Sphaerium fabale* (Prime, 1852) [*S. simile* (Say, 1816), *S. striatinum* (Lamarck, 1818)]] was among the best supported tip clades in our gene trees (Figs 1–3), corroborating earlier findings based on allozymes (Hornbach, McLeod & Guttaman, 1980; Hornbach *et al.,* 1980) and 16S gene sequences (Cooley & Ó Foighil, 2000). Interestingly, the European *S. rivicola* (type species of the subgenus *Sphaeriastrum* Bourguignat, 1854 was robustly sister to the North American taxa in the ITS1 trees (Fig. 2). Although we were unsuccessful in generating 16S sequences for *S. rivicola*, we did obtain sequences for another mt gene (COI, data not presented here) which also placed it sister to

North American *Amesoda* (Lee, 2001), as did the morphological analysis of Korniushin & Glaubrecht (2002). Our data is in full agreement with their reassignment of *S. rivicola* to *Amesoda*, although in our system it is at the subgeneric rank.

Subgenus *Sphaerinova* Iredale, 1943 (Type species: *Cyclas tasmanica* Tenison Woods, 1876)

Our two antipodean taxa, *Sphaerium tasmanicum* and *S. novaezelandia*, consistently formed a well-defined robust clade which was nested among the other asynchronous brooders and, in the ITS1-containing analyses, was weakly sister to *Sphaerium s.s.* (Figs 2 and 3). These results support Kuiper's (1966) proposal to place them in a separate *Sphaerium* subgenus, *Sphaerinova*, but not Korniushin's (2000) designation of *Sphaerinova* as a *Musculium* subgenus. More recently, Korniushin & Glaubrecht (2002) also found that these two taxa formed a weakly supported (BS < 50) clade in their morphological dataset, but unjustifiably included polytomous *Musculium* taxa from a variety of continents in their conception of *Sphaerinova* (see above).

Subgenus *Herringtonium* Clarke, 1973 (Type species: *Cyclas occidentalis* Prime, 1856)

The *Sphaerium* subgenus *Herringtonium* was formed as a monotypic entity to accommodate the North American species *Sphaerium occidentale*, which has a mix of morphological, reproductive and ecological features that was not deemed to convincingly match any of the above subgeneric groupings (Clarke, 1973). As was the case for *S. transversum*, *S. occidentale*'s topological placement shows no consensus among 16S (Fig. 1), ITS1 (Fig. 2) or morphological datasets [where it was part of a poorly supported paraphyletic grade of *Musculium* and *Sphaerium* taxa (Korniushin & Glaubrecht, 2002)]. However, in one of these datasets (ITS1) the node supporting *S. occidentale* is impressively robust (Fig. 2; BS = 100, DI = 5) and this support carries through into the combined MP and ML analyses (Fig. 3).

The sister taxon occupying this ITS-supported node with *S. occidentale* is another North American taxon, *S. rhomboideum*, and in the combined analyses (Fig. 3) they are positioned basally within the clade of asynchronous brooders, sister to all other *Sphaerium* taxa. Detailed comparison of their ITS1 sequences shows them to be compellingly similar, differing only in two point mutations and in the presence of two minor inferred indels. Korniushin & Glaubrecht (2002) placed *S. rhomboideum* in *Sphaerium s.s.*, sister to *S. nucleus*. However, this topological placement is clearly incongruent with our results and it adds 16 steps to the combined MP analysis tree (Fig. 3). Our new data strongly suggest that *S. rhomboideum* be reassigned to the subgenus *Herringtonium*, and we anticipate that additional data from suitably slowly evolving nuclear genes such as 28S rDNA would act to corroborate this placement.

In summary, phylogenetic relationships of the Sphaeriinae were reconstructed using a nuclear (ITS1) and a mitochondrial (16S) ribosomal gene sequence data. All phylogenetic analyses of individual and combined datasets recovered a paraphyletic *Pisidium* and a derived clade of asynchronous brooding *Sphaerium*/*Musculium* taxa. Utilizing robustly supported clades in our gene trees, current sphaeriine taxonomy was revised. Five major monophyletic lineages, *Afropisidium*, *Odhneripisidium*, *Pisidium*, *Cyclocalyx* and *Sphaerium*, were recognized at the generic level. In addition, a number of subgeneric level groups were recovered in *Sphaerium*: *Herringtonium*, *Sphaerium s.s.*, *Sphaerinova*, *Amesoda*, and *Musculium* together with one unassigned species, *S. transversum*.

Although our dataset provides valuable new insights in sphaeriinid evolution and systematics, data (including morphological characters) from additional taxa (especially *Neopisidium*, *Afropisidium* and *Odhneripisidium*), and from slowly evolving genes, are required to flesh out basal phylogenetic relationships among major sphaeriine lineages. A comprehensive understanding of sphaeriid evolution and cladogenesis awaits the incorporation of equivalent datasets from the sister Euperinae clade and the identification of convincing marine outgroup(s).

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