PRONOUNCED KARYOLOGICAL DIVERGENCE OF THE NORTH AMERICAN CONGENERS *SPHAERIUM RHOMBOIDEUM* AND *S. OCCIDENTALE* (BIVALVIA: VENEROIDA: SPHAERIIDAE)

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(Received 8 November 2006; accepted 25 June 2007)

ABSTRACT

Chromosome sets of two North American sphaeriid species, *Sphaerium rhomboideum* Say, 1822 and *S. occidentale* Lewis, 1856, were studied using conventional Giemsa staining and karyometric analysis. Pronounced karyological divergence of congeners was revealed. The diploid number of 2n = 44 was reported for *S. rhomboideum* and this is the first record of a diploid species in the highly polychromosomic Nearctic sphaeriid fauna. The karyotype was characterized by medium-sized and small chromosomes, which decreased in size gradually from 5.77 to 1.9 μm. Biarmed chromosomes with medially and submedially located centromeres predominated, but six pairs of subtelo-telocentric elements were also observed in the karyotype. The estimated mitotic chromosome number for *S. occidentale* ranges from 189 to 213, but most of the cells examined contained about 204–209 chromosomes. A first attempt to karyotype a polyploid sphaeriid was made. It was revealed that the comparatively large and middle-sized chromosomes could be grouped in four, so the karyotype presumably evolved through tetraploidization. The small chromosomes formed the large fraction, about 137. Due to their similar and indistinct morphologies, it was impossible to arrange them into subgroups with confidence. Revealed karyological characteristics are discussed with reference to the existing phylogenetic interpretations of the evolutionary history of the Sphaeriinae.

INTRODUCTION

Although many animals exist as natural polyploids (reviewed in Lokki & Saura, 1980; Schultz, 1980; Otto & Whitton, 2000), the general perception is that, unlike plants, polyploidization is too rare to have been a significant factor in animal evolution (Orr, 1990; Mable, 2004). Polyploidy is thought to be particularly rare in bivalve molluscs (Nakamura, 1985; Thiriot-Qiuévreux, 2002) with the best-known cases being restricted to clonal lineages of two genera: *Corbicula* (Komaru & Konishi, 1999; Park *et al*., 2000; Qiu, Shi & Komaru, 2001) and *Lasaea* (Thiriot-Quiévreux *et al*., 1988; Ó Foighil & Thiriot-Qiuévreux, 1991, 1999). However, recent cytogenetic studies of sphaeriine clams, a subfamily of the exclusively freshwater Sphaeriidae, have revealed the presence of exceptionally variable mitotic chromosome numbers, from 30 to 247 (Table 1), among diverse constituent taxa (Baršienė, Tapia & Baršytė, 1996; Burch, Park & Chung, 1998; Lee, 1999; Park *et al*., 2002; Lee & Ó Foighil, 2002; Jara-Seguel, Peredo & Parada, 2005; Petkevičiūtė, Stunžėnas & Stanevičiūtė, 2006). Apart from the European *Sphaerium corneum* (Linnaeus, 1758) diploid species complex (Keyl, 1956; Petkevičiūtė *et al*., 2006), all sphaeriine species studied to-date have chromosome numbers (Table 1) that greatly exceed the normal veneroid bivalve range of 24–48 diploid chromosomes (Nakamura, 1985; Thiriot-Qiuévreux, 2002). Although these extraordinary karyological complements strongly suggest that pronounced polyploidization is prevalent in Sphaeriinae, the evolutionary origins of genome amplification in this clade remain obscure, as does the actual levels of ploidy they exhibit.

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Polyploidy may originate within a single species (autopolyploidy), result from hybridization among partially cross-fertile ancestral species (segmental allopolyploidy), or result from hybridization among almost completely cross-sterile ancestral species (genomic allopolyploidy) (Thompson & Lumaret, 1992; Gaut & Doebley, 1997). The latter case is the one most frequently encountered in natural populations (Soltis & Soltis, 1993), whereas autopolyploids are extremely rare in animals (Dufresne & Hebert, 1994). Lee & Ó Foighil (2002) investigated the temporal interrelationships of inferred genome duplication and speciation events among some North American polyploid sphaeriines by constructing single-copy nuclear-gene allelic trees based on within-individual and among-species variation in the 6-phosphogluconate dehydrogenase (PGD) locus. Their results were consistent with an early genome duplication, predating the divergence of the genotyped members of three *Sphaerium* subgenera: *Amesoda* [*S. striatinum* (Lamarck, 1818) and *S. simile* (Say, 1817)], *Herringtonium* (*S. occidentale*) and *Musculium* [*S. securis* (Prime, 1852)]. While interesting, this conclusion in itself was insufficient to reveal comprehensively the evolutionary roots of genome amplification in these species, because their chromosome complements (Table 1) suggest multiple episodes of genomic amplification and the PGD dataset lacked diploid sphaeriinid reference taxa.

Advancing our knowledge of sphaeriine genome amplification processes will hinge on the identification of robust sister relationships among taxa differing in chromosome complements, especially those involving diploid and polyploid species. Lithuanian populations of the diploid Eurasian species *S. corneum* contain two karyotypic forms, 2n = 30 and 36 (Petkevičiūtė *et al*., 2006), but the chromosomal composition of their sister taxa, *S. nucleus* and *S. baicalense* (Lee & Ó Foighil, 2003; Petkevičiūtė *et al*., 2006), are unknown (Fig. 1).

Journal of Molluscan Studies (2007) ***73****: 315–321. Advance Access Publication: 23 October 2007* *doi:10.1093/mollus/eym025*

Table 1. Chromosome numbers in the Sphaeriinae.

Species	Locality	Chromosome number		References
		n	2n × n	
Sphaerium (*Sphaerium*) *corneum*	Germany	18	36	Keyl (1956)
	Lithuania		30	Petkevičiūtė *et al.* (2006)
	Lithuania		36	Petkevičiūtė *et al.* (2006)
S. (*Amesoda*) *simile*	USA		>100	Lee & Ó Foighil (2002)
S. (*A.*) *striatinum*	USA	~76	~152	Lee (1999)
S. (*Herringtonium*) *occidentale*	USA		~209	Burch *et al.* (1998)
S. (*Musculium*) *argentinum*	Chile		~130	Jara-Seguel *et al.* (2005)
S. (*M.*) *securis*	USA		~247	Burch *et al.* (1998)
Cyclocalyx adamsi	USA		>100	Lee & Ó Foighil (2002)
C. casertanum	Spain		~150, 180	Baršienė *et al.* (1996)
	USA		~190	Burch *et al.* (1998)
C. compressum	USA		>100	Lee & Ó Foighil (2002)
C. (?) *coreanum*	Korea	95	190	Park *et al.* (2002)
Pisidium dubium	USA		>200	Lee & Ó Foighil (2002)

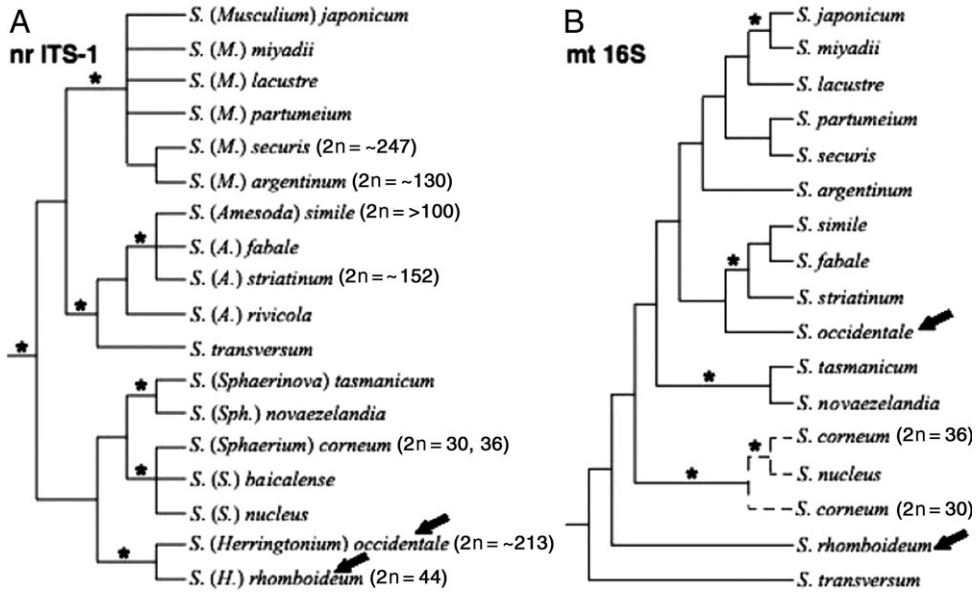

Figure 1. Molecular gene trees for sequential-brooding *Sphaerium* taxa proposed by Lee & Ó Foighil (2003). **A.** Nuclear gene tree based on ribosomal first internal transcribed spacer (ITS-1) sequences. **B.** Mitochondrial tree based on large ribosomal subunit (16S) gene fragments. Mitotic chromosome numbers are provided for cytogenetically studied taxa. Branches supported by parsimony bootstrap values >70 are indicated by asterisks (*). Mitochondrial sister relationships among two karyotipic forms of *S. corneum* (2n = 30 and 36) and *S. nucleus* are inferred from Petkevičiutė *et al.* (2006) and presented as dashed line. Note that two North American taxa, *S. occidentale* and *S. rhomboideum*, form a robust monophyletic clade in ITS-1 tree but are not sister in mitochondrial 16S tree.

In the present study, we karyotyped two North American taxa, *Sphaerium occidentale* and *S. rhomboideum*, that displayed a robust and exclusive sister relationship for a nuclear ribosomal marker (ITS-1) but, interestingly, were not sister taxa for a mitochondrial (mt) ribosomal marker (16S RNA) (Fig. 1; Lee & Ó Foighil, 2003). A chromosome count of about 209 has been recorded for *S. occidentale* (Burch *et al.*, 1998) and our karyotype of this species is the first such for a polyploid sphaeriinid. *Sphaerium rhomboideum*'s chromosomal complement has not been previously studied and we found it to be diploid, the first such record for a New World sphaeriine and one that represents a novel focal point for investigating the evolution of polyploidy in these taxa.

MATERIAL AND METHODS

Specimens of *Sphaerium rhomboideum* and *S. occidentale* were collected in August 2005 from vernal ponds bordering Douglas Lake (at Sedge Point and Grapevine Point, respectively) on the University of Michigan's Biological Station grounds in Pellston, Michigan, USA. Voucher specimens of the populations from which chromosomes were studied have been deposited in the collections of the Museum of Zoology, University of Michigan (catalogue numbers UMMZ300355 and UMMZ300354). In order to verify their phylogenetic positions relative to the previous molecular study (Lee & Ó Foighil, 2003), four individuals studied for chromosome complements were chosen from each

species, and the target fragment of mt large ribosomal subunit (16S) and the entire nuclear ribosomal first internal transcribed spacer (ITS-1) were sequenced following the methods described in Lee & Ó Foighil (2003).

Initial phases of chromosome preparation were completed as soon as possible after the animals were collected and identified. To obtain metaphases, animals were maintained in 0.01% colchicine in lake water for 3–4 h. The bodies were removed from the shells under a dissecting microscope and placed in distilled water for 40–50 min of hypotonic treatment at room temperature. Tissues were fixed by three incubations of 20 min each in a freshly mixed modified Carnoy's fixative (three part ethyl alcohol and one part glacial acetic acid). Fixed tissues were kept refrigerated until they could be processed in the laboratory. Each slide preparation was made from one individual using an air-drying technique. This involved placing a small piece of fixed tissue on a clean microscope slide in a few drops of 50% acetic acid, smearing dissociated cells onto the slide and drying by heating to 40–50°C with an alcohol lamp. Preparations were then treated with 1 N HCl for 10–15 min, rinsed three times in distilled water, and stained for 30 min with 4% Giemsa solution made up in phosphate buffer, pH 6.8.

The chromosome preparations were examined with a Zeiss (Amplival) microscope using a 100× oil immersion objective. Chromosome spreads were karyotyped from photographic prints, arranging the chromosomes based on type (centromere position) and in descending order of size. Ambiguities in karyotypes were resolved by viewing cells directly with the microscope. Chromosome arm lengths (short arm, p; long arm, q) were measured. For each chromosome pair, mean and standard deviations of absolute length ($p + q$), relative length ($(p + q)$/ total haploid length/100) and centromeric index ($100 \times p/(p + q)$) were calculated in a Microsoft Excel spreadsheet. Centromere position nomenclature follows that of Levan, Fredga & Sandberg (1964). In situations where the standard deviation of the centromeric index was at the borderline between two chromosome types, the nomenclature for both was given.

RESULTS

The present molecular characterizations confirmed that the studied populations of both species represent the same phylogenetic lineages as defined by Lee & Ó Foighil (2003). All individuals characterized for nuclear ITS-1 had an identical genotype either to AY093538 (*Sphaerium rhomboideum*) or to AY093542 (*S. occidentale*) obtained in the previous study (Lee & Ó Foighil, 2003). While two individuals of *S. occidentale* had the same 16S haplotype as the previously studied samples (AF152046), a single nucleotide insertion was observed in the other two individuals. The 472-nucleotide 16S fragment of *S. rhomboideum* differed from the previously generated sequence (AF152038) by only two substitutions. All newly obtained DNA sequences have been deposited in GenBank (DQ986372, DQ986373).

Sphaerium rhomboideum

Fourteen processed specimens of *S. rhomboideum* generated a total of 109 mitotic metaphase plates in which the chromosomes were sufficiently spread to be individually distinguished. Among them, 86 metaphases (78.9%) contained 44 chromosomes in diploid sets. Other values, most often lower than modal (2n = 42 or 43), represent aneuploidies or (more likely) a loss of chromosomes during processing, a technical artifact commonly encountered in the slide-preparation method used. Four triploid and one tetraploid cell were encountered. The 12 best-spread metaphase plates containing 44 chromosomes, collectively obtained from six specimens of *S. rhomboideum*, were used for karyotyping. A representative karyotype is shown in Figure 2. Chromosome measurements were made from 10 metaphases (Table 2). Chromosomes were medium-sized: their mean absolute lengths ranged from 5.77 to 1.90 μm. The 22 chromosome pairs maintained small length differences between successive pairs (<0.60 μm) and did not form distinguishable size classes. The mean total length of the haploid complement reached 86.16 μm. Biarmed elements predominated in the karyotype. According to the centromere position, 12 chromosome pairs (chromosome number 1, 2, 5, 8, 9, 11, 13, 14, 16, 18, 20 and 22) were classified as metacentric; pair 4 was submetacentric; pair 7 was intermediate submeta-metacentric; pairs 6, 10, 12 and 15 were subtelo-telocentric; pairs 3 and 17 were subtelocentric; and pairs 19 and 21 were submeta-subtelocentric.

Sphaerium occidentale

Mitotic metaphase spreads of *S. occidentale* contained much larger numbers of chromosomes than those of *S. rhomboideum*. They were difficult to enumerate accurately due to the high frequency of overlapping chromosomes in the preparations and we could confidently count the chromosomes of only nine cells obtained from a total of four individual clams. Among the cells studied, we counted 189, 191, 197 and 213 chromosomes in each of the four cells, 204–207 in three cells and 208–209 in two cells. Thus, the estimated mitotic chromosome number for *S. occidentale* ranges from 189 to 213. Figure 3 shows the karyotype generated from a mitotic metaphase with 213 chromosomes. In the karyotype, we have separated nine groups of chromosomes based on similarities of size and morphology. Group I includes the 12 largest metacentric chromosomes and the absolute length of the largest elements reached 5 μm. Group II is comprised of 12 large elements with subterminally localized centromeres and Group III includes four comparatively large chromosomes with a submetacentric type of structure. Medium-sized chromosomes were arranged in Groups IV–VI, including 12 metacentric, 20 submetacentric and 16 chromosomes with subterminal centromeres, respectively. Thus, there were 76 comparatively large or medium-sized chromosomes in this set. It is notable that the chromosomes within Groups I–VI fall into sets of four chromosomes each. The smallest chromosomes were arranged into Groups VII–IX, containing 88 metacentric, 28 submeta-subtelocentric and 21 dot-like elements of unclear morphology. Thus, the fraction of small chromosomes contained 137 elements. The morphology of the smallest chromosomes (<1 μm) was not distinct in some cases and therefore some ambiguities remain.

DISCUSSION

The diploid number 2n = 44 reported here for *Sphaerium rhomboideum* is unusual in that it has not been recorded in any other bivalve species. Diploid chromosome number of bivalves range from 2n = 12 to 48, with 2n = 38 being the most frequent number, found chiefly in Veneroida (see Nakamura, 1985; Thiriot-Qiuévreux, 2002). The discovery of diploidy in *S. rhomboideum* is particularly interesting as this is the first record of a diploid species in the Nearctic sphaeriid fauna.

There is no obvious concordance between the chromosomal complements of *S. rhomboideum* and its diploid Eurasian congener *S. corneum*. Two karyotypic forms of *S. corneum* differing in chromosome number (2n = 30 and 36) and chromosome morphology were recently discovered in Lithuania (Petkevičiūtė *et al.*, 2006). The karyotype with 2n = 30 consisted of biarmed, metacentric and meta-submetacentric pairs although variable numbers (0–10) of small metacentric supernumerary (B) chromosomes were detected in some cells. The karyotypic

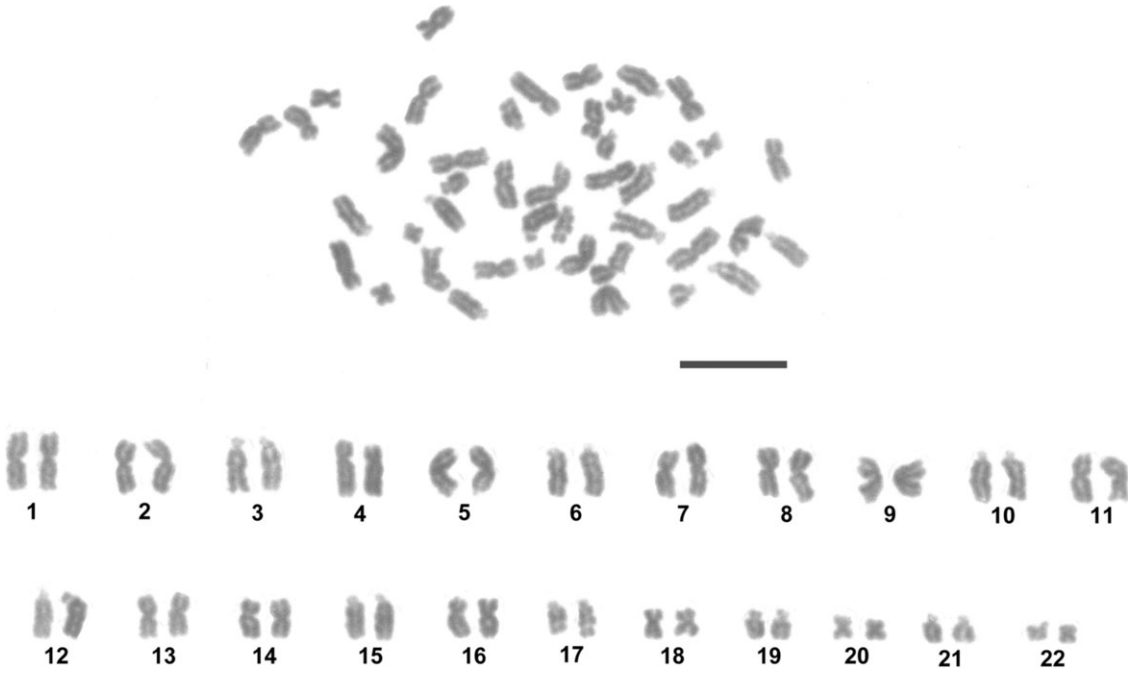

Figure 2. Mitotic metaphase and karyotype of *Sphaerium rhomboideum*, 2n = 44. Scale bar = 10 μm.

form with 2n = 36 was characterized by a prevalence of biarmed elements, but telocentric and subtelocentric chromosomes also were present in this karyotype. Petkevičiūtė *et al*., 2006 concluded that Robertsonian fusions might have been involved in formation of two karyotypic forms of *S. corneum*. The karyotype of *S. rhomboideum* is broadly similar to *S. corneum* in its high number of biarmed chromosomes. However, the many karyological distinctions of *S. rhomboideum* and *S. corneum* probably result from a long period of evolutionary divergence; they are not sister taxa (Fig. 1; Lee & Ó Foighil, 2003). Despite the higher *S. rhomboideum* diploid number, the mean total length of its haploid complement does not exceed that of either karyotypic form of *S. corneum*; *S. corneum* chromosomes are evidently larger.

Burch *et al*. (1998) reported a high mitotic chromosome number, about 209, for *S. occidentale*. Our chromosome counts, 189–213, confirmed this observation and most cells examined

Table 2. Measurements (means ± SD) and classification of chromosomes of *Sphaerium rhomboideum*.

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Classification*
1	5.77 ± 1.18	6.70 ± 0.50	46.39 ± 3.04	m
2	5.17 ± 1.00	6.01 ± 0.37	44.05 ± 4.64	m
3	5.11 ± 1.22	5.89 ± 0.48	15.60 ± 3.41	st
4	4.84 ± 0.94	5.62 ± 0.30	34.93 ± 4.97	sm
5	4.79 ± 0.98	5.55 ± 0.29	45.05 ± 4.03	m
6	4.69 ± 1.00	5.43 ± 0.32	12.78 ± 3.20	st–t
7	4.53 ± 0.92	5.26 ± 0.29	36.99 ± 5.60	sm–m
8	4.51 ± 0.96	5.22 ± 0.24	41.90 ± 4.26	m
9	4.46 ± 103	5.16 ± 0.37	43.31 ± 3.40	m
10	4.20 ± 0.74	4.89 ± 0.27	13.10 ± 3.90	st–t
11	4.08 ± 0.85	4.72 ± 0.19	42.53 ± 4.73	m
12	3.94 ± 0.89	4.55 ± 0.22	12.94 ± 3.94	st–t
13	3.85 ± 0.93	4.44 ± 0.36	41.21 ± 3.21	m
14	3.70 ± 0.75	4.29 ± 0.23	43.81 ± 2.82	m
15	3.62 ± 0.69	4.22 ± 0.40	14.81 ± 3.41	st–t
16	3.33 ± 0.63	3.87 ± 0.19	41.24 ± 2.92	m
17	3.20 ± 0.72	3.71 ± 0.26	20.35 ± 3.50	St
18	2.88 ± 0.54	3.34 ± 0.17	44.58 ± 3.22	m
19	2.84 ± 0.43	3.33 ± 0.36	26.40 ± 5.27	sm–st
20	2.46 ± 0.37	2.89 ± 0.36	43.06 ± 4.09	m
21	2.29 ± 0.43	2.67 ± 0.24	26.12 ± 3.55	sm–st
22	1.90 ± 0.25	2.23 ± 0.25	46.37 ± 4.11	m

*m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric chromosome.

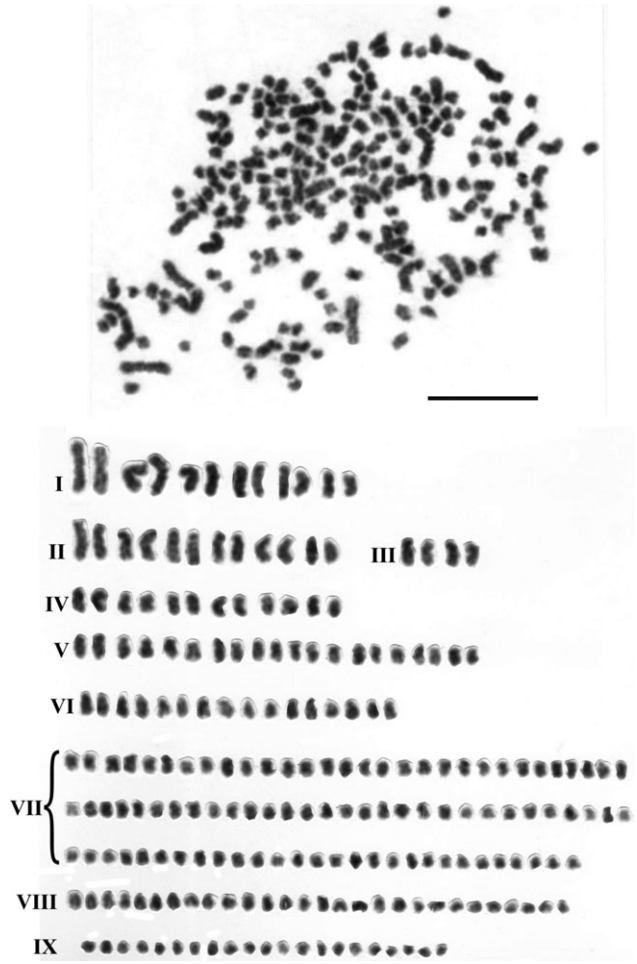

Figure 3. Mitotic metaphase and karyotype with 213 chromosomes of *Sphaerium occidentale*. Scale bar = 10 μm.

contained about 204–209 chromosomes. No details on the karyotype structure of any polyploid sphaeriid species have previously been published nor have ploidy levels been determined. Polyploidy must refer to a haploid number and, if we consider a hypothetical ancestor with a haploid number of 19 (the common haploid number among Veneroida), *S. occidentale* with about 209 chromosomes should be considered as 11n and chromosomes should occur in multiples of 11. This was not the case. The largest and medium-sized chromosomes of *S. occidentale* clustered into subgroups of four on the basis of shared size and morphology.

A striking feature of the *S. occidentale* karyotype was the large fraction of small chromosomes – 137 or 64.2% of the total chromosome number. It was impossible for us to arrange with confidence the smallest chromosomes into subgroups due to their similar and indistinct morphologies. The significance of such an enormous number of tiny chromosomes in *S. occidentale* cells is obscure, but it is difficult to envisage them all as representing supernumeries. In contrast, *S. rhomboideum* chromosomes decreased in size fairly gradually, size classes were not clearly distinguishable, and only the three last pairs (13.6% of the total number) could be characterized as being relatively small.

The utility of karyotypic data in determining phylogenetic relationships depends on three main factors: the taxonomic coverage of the database, the level of karyological detail available per taxon and the underlying pattern of chromosome evolution. Unfortunately, the existing sphaeriine karyological dataset is quite limited, due to spotty taxonomic coverage and to the availability of chromosome counts only for polyploid taxa (Fig. 1; Table 1). The present study represents the first attempt to karyotype a polyploid sphaeriine and the results obtained indicate that the formation of the *S. occidentale* karyotype (Fig. 3) was more complex than the amplification of one particular haploid set. Large and middle-sized chromosomes could be grouped in four, so this karyotype presumably evolved through tetraploidization. However, the available chromosomal data are insufficient to determine how this came about, i.e. is *S. occidentale* an autopolyploid or an allopolyploid? Another unresolved issue concerns the origins of the large fraction of small chromosomes present in *S. occidentale*. They could be, at least partly, explained by the accumulation of B chromosomes, but the involvement of the other possible mechanisms cannot be ruled out at present. A similar phenomenon, the appearance of large number of small-sized chromosomes in polyploid cells of some specimens of *Mytilus trossulus*, was documented as the possible consequence of a neoplastic condition (González-Tizón *et al*., 1999).

Sphaeriine chromosome evolutionary relationships could be clarified by placing taxon-specific karyotype features on phylogenetic tree topologies, but recent phylogenetic studies (Dreher-Mansur & Meier-Brook, 2000; Cooley & Ó Foighil, 2000; Korniushin & Glaubrecht, 2002; Lee & Ó Foighil, 2003; Lee, 2004) have produced conflicting results at both inter and intra-generic levels. The inter-generic issues, centering on whether or not the sequentially-brooding taxa (*Sphaerium* and *Musculium*) are monophyletic, have been resolved in favour of sequential-brooder monophyly (Lee & Ó Foighil, 2003; Lee, 2004). However, topological relationships among some sequential brooding taxa, including *S. rhomboideum* and *S. occidentale*, are labile, in that different datasets (both molecular and morphological) yield significantly incongruent topologies (Korniushin & Glaubrecht, 2002; Lee & Ó Foighil, 2003).

Lee & Ó Foighil (2003) used a combined nuclear and mitochondrial gene-tree topology to revise sphaeriine taxonomy and one of the more robust (bootstrap = 98; decay index = 6), and topologically distinct, sister relationships they recovered within the sequential-brooding clade involved *S. rhomboideum* and *S. occidentale*. They consequently placed both taxa in the *Sphaerium* subgenus *Herringtonium* (Clarke, 1973), originally formed as a monotypic entity for *S. occidentale* because its mix of morphological, reproductive and ecological features were deemed an unconvincingly match to other sequential-brooder groupings (Clarke, 1973). Our novel karyological result, showing that these two taxa differ spectacularly in chromosomal repertoires (Figs 2, 3), was therefore unexpected, because the diploid condition found in *S. rhomboideum* presumably represents a plesiomorphic condition, whereas the polyploid *S. occidentale* genome is likely to be apomorphic.

It is important to emphasize that the phylogenetic signal for the inferred sister relationship of *S. rhomboideum* and *S. occidentale* stemmed solely from the nuclear ribosomal (ITS-1; Fig. 1A) marker, for which these two species differed by only two point mutations and two minor indels (Lee & Ó Foighil, 2003). However, mt (16S; Fig. 1B; Lee & Ó Foighil, 2003) and morphological (Korniushin & Glaubrecht, 2002) datasets did not recover this sister relationship. An obvious question is just how did these two clam species, differing so markedly in their chromosomal compositions (Figs 2, 3), end up sharing such similar nuclear ribsomal (ITS-1) genotypes?

One possible explanation is that *Sphaerium occidentale* is an allopolyploid and that diploid *S. rhomboideum* is an extant representative of one of its parental lineages. Allopolyploidization appears to be the most prevalent mechanism of genome duplication (Soltis & Soltis, 1993; Dufresne & Hebert, 1994) and such hybrid genomes may undergo dramatic chromosomal reorganization prior to stabilization (Song *et al*., 1995). Due to their

predominantly uniparental inheritance, mitochondrial genealogies are insufficient to reconstruct ancestral reticulations and nuclear ribosomal genealogies are also problematic in such cases because of their propensity for concerted evolutionary processes that can erode the signature of biparental species ancestry (Hillis *et al*., 1991; Roelofs *et al*., 1997). Single-copy nuclear genes may best preserve a reticulate signal (Cronn, Small & Wendel, 1999) and Lee & Ó Foighil (2002) found potential evidence for this in a number of polyploid sequential-brooding North American sphaeriines, including *S. occidentale*, in which two highly divergent and phylogenetically distinct PGD allelic clades were co-expressed by individual clams. The PGD results were interpreted as being consistent with an ancient genome duplication that predated cladogenesis of the studied polyploid taxa (Lee & Ó Foighil, 2002). However, this is inconsistent with our novel karyological finding that *S. rhomboideum*, the inferred sister taxon (based on ITS-1 sequences; Fig. 1A) of the highly polyploid *S. occidentale*, is diploid.

The absence of PGD data from diploid sequential brooders, such as *S. rhomboideum* (this study) and *S. corneum* (Keyl, 1956; Petkevičiūtė *et al*., 2006) did not allow Lee & Ó Foighil (2002) to test for evolutionarily-recent allopolyploidization events among their North American study taxa, including *S. occidentale*. We plan to amend this shortcoming by genotyping these diploid sphaeriine sequential-brooding taxa for the PGD marker. Based on Lee & Ó Foighil's (2003) ITS-1 topology, and assuming that *S. occidentale* is an allopolyploid, we predict that *S. rhomboideum* PGD alleles will nest within one of the two divergent clades present for this locus in polyploid, sequentially-brooding North American congeners (Lee & Ó Foighil, 2002), where they will be sister to that clade's *S. occidentale* alleles.

Sphaeriidae are ubiquitous in freshwater ecosystems (Herrington, 1962; Clarke, 1973; Kuiper, 1983) and represent one of the major molluscan radiations into freshwater habitats (Kuiper, 1983; McMahon, 1991). Recent morphological (Dreher-Mansur & Meier-Brook, 2000; Korniushin & Glaubrecht, 2002; Lee, 2004) and molecular (Cooley & Ó Foighil, 2000; Park & Ó Foighil, 2000; Lee & Ó Foighil, 2002, 2003) phylogenetic studies have generated conflicting interpretations of the evolutionary history of the cosmopolitan subfamily Sphaeriinae. An unknown fraction of this incongruence may stem from latent patterns of pronounced genome amplification. Our discovery of the first example of a North American diploid species represents a promising initial opportunity to investigate meaningfully the importance of polyploidization in sphaeriine cladogenesis and it highlights the need for more extensive karyological characterization of these taxa.

ACKNOWLEDGEMENTS

This project was funded by the United States National Research Council's Twinning Program for 2005–2006. Special thanks to J.B. Burch for assistance in collecting samples. We also thank the University of Michigan Biological Station, Pellston, Michigan, for making laboratory and living facilities as well as field sites available.

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