

**CHAPTER 3**  
**6-PHOSPHOGLUCONATE DEHYDROGENASE (PGD) ALLELE PHYLOGENY**  
**IS INCONGRUENT WITH A RECENT ORIGIN OF POLYPLOIDIZATION**  
**IN SOME NORTH AMERICAN SPHAERIIDAE**

**Introduction**

Polyploidization is much more common in plants than in animals (Muller, 1925; Orr, 1990; Dufresne and Herbert, 1994) and the process of genome duplication is thought to have played a remarkably influential role in plant evolution (Soltis and Soltis, 1993, 1999; Leitch and Benett, 1997). Among animals, polyploidization is particularly rare in bivalve mollusks (Nakamura, 1985; Okamoto and Arimoto, 1986; Ó Foighil and Thiriot-Quévieux, 1999), although it is routinely induced in cultured species (Eudeline *et al.*, 2000). Surprisingly, recent cytogenetic studies suggest that pronounced genome amplification may be prevalent in the cosmopolitan freshwater bivalve family Sphaeriidae (Park, 1992; Baršienė *et al.*, 1996; Burch *et al.*, 1998; Lee, 1999). A number of phylogenetic studies of the Sphaeriidae have recently been published based on morphological (Dreher-Mansur and Meier-Brook, 2000) and molecular (Park and Ó Foighil, 2000; Cooley and Ó Foighil, 2000) data sets. None of these papers, however, addressed the evolutionary origins of genome duplication in sphaeriids and what role this process has played in their cladogenesis. This is the aim of the study.

The Sphaeriidae first appeared in Cretaceous freshwater habitats (Keen and Dance, 1969) and are often the dominant benthic organisms in streams and ponds (Eckblad *et al.*, 1977) where they play a key role in energy and nutrient cycling

(Hornbach *et al.*, 1984; Lopez and Holopainen, 1987). A single diploid species is known [the Eurasian *Sphaerium corneum* (Linnaeus),  $2n = 36$  (Keyl, 1956)]. However, all other typed taxa, including members of the three cosmopolitan genera (*Sphaerium*, *Musculium* and *Pisidium*), exhibit exceptionally high and variable mitotic chromosome numbers, ranging from 152 to 247 (Park, 1992; Baršienė *et al.*, 1996; Burch *et al.*, 1998; Lee, 1999). Spermatogenetic meiosis has been observed in diploid *S. corneum* (Keyl, 1956), as well as in two polyploid taxa, *P. coreanum* (Park, 1992) and *S. striatinum* (Lee, 1999), indicating that at least some of the polyploids may reproduce sexually.

Our understanding of the evolutionary dynamics of genome duplication stems primarily from botanical research (Soltis and Soltis, 1993, 1999; Leitch and Bennett, 1997; Wendel, 2000) and this body of knowledge has informed the approach I took to investigate the evolutionary origins of polyploidization in the Sphaeriidae. Genome duplication may occur within a species (autopolyploidy), or result from hybridization among ancestral species (allopolyploidy) (Thompson and Lumaret, 1992; Gaut and Doebley, 1997). Allopolyploidization appears to be the most prevalent method of genome duplication (Soltis and Soltis, 1993; Dufresne and Hebert, 1994) and such hybrid genomes may undergo remarkably rapid and dramatic rearrangements prior to stabilization (Bennett *et al.*, 1992; Kenton *et al.*, 1993; Song *et al.*, 1995). If sphaeriid polyploidization stems from reticulation events, mitochondrial gene trees will be insufficient, and perhaps misleading, in reconstructing sphaeriid phylogeny due to their presumed uniparental inheritance pattern. Such shortcomings can be surmounted by cross-referencing sphaeriid mitochondrial phylogenies (Cooley and Ó Foighil, 2000) with nuclear gene trees. However, nuclear genes duplicated by polyploidization may experience diverse fates including gene silencing, homogenization, or independent evolution and differentiation (Wendel, 2000).

Although parental complements of highly repetitive genes (such as the rRNA genes) may persist in some asexual and/or evolutionarily young allopolyploids (Sang *et al.*, 1995; Campbell *et al.*, 1997; Hugall *et al.*, 1999; Widmer and Baltisberger, 1999), they typically become homogenized through concerted evolutionary processes (Hillis *et al.*, 1991; van Houten *et al.*, 1993; Wendel *et al.*, 1995; Roelofs *et al.*, 1997; Fuertes Aguilar *et al.*, 1999). Cronn *et al.* (1999) provided evidence for independent evolution of duplicated low-copy genes and predicted that single-copy genes may best retain their evolutionary independence following polyploidization. I chose the single-copy gene route, targeting 6-Phosphogluconate Dehydrogenase (PGD), a key enzyme in the pentose-phosphate biochemical pathway, which has been the subject of extensive biochemical and population genetic characterization (Scott and Lucchesi, 1991; Begun and Aquadro, 1991, 1994). In the Sphaeriidae, observed polyploid levels are very high (8-13n; Lee, 1999), raising the distinct possibility of severe pseudogene complications, even for a single-copy target gene. To minimize this potential confounding factor, I chose to amplify only from expressed alleles using a reverse-transcriptase-polymerase chain reaction (RT-PCR) protocol.

Expressed allelic repertoires of the target PGD fragment were characterized for seven North American polyploid species, belonging to three cosmopolitan genera, and analyzed phylogenetically. Assuming that duplicated PGD alleles have maintained their phylogenetic independence and expression (*i.e.*, phylogenetic signal is not obscured by recombination and/or gene silencing), I predicted that the topology of intra-individual and inter-species sister alleles recovered will reflect the temporal interrelationship of genome duplication and speciation events among the study taxa. For instance, an individual's allelic repertoire will form a species-specific clade if evolutionarily recent post-speciation polyploidization occurred (Figure 3-1a). On the other hand, intra-individual allelic repertoires will be polyphyletic and exhibit cross-species sister relationships if genome duplication predated speciation events among the study taxa (Figure 3-1b). Although

modulated by evidence for limited recombination and unbalanced gene expression patterns, the results are consistent with an ancient genome duplication, predating the divergence of the investigated *Sphaerium/Musculium* North American taxa.

## Materials and Methods

### Collection of Specimens

Seven polyploid species of sphaeriids (subfamily Sphaeriinae), chosen to be representative of North American systematic diversity (Cooley and Ó Foighil, 2000), were collected in Michigan, USA, from June 1997 to July 1999 (Table 3-1). The chromosome numbers of three of these species, *Musculium securis* ( $2n = 247$ ), *Sphaerium occidentale* ( $2n = 209$ ) and *S. striatinum* ( $2n = 152$ ), have previously been determined (Burch *et al.*, 1998; Lee, 1999). Mitotic chromosomes of *Pisidium adamsi*, *P. compressum*, *P. dubium* and *S. simile* were observed using an acetic-orcein gonadal squash method [described in Lee (1999)], and the numbers in each case were well over 100 (see Table 3-1 for the estimation). Tissue samples of *Eupera cubensis* (subfamily Euperinae, of unknown ploidy level) were collected in Cuba by Mary Yong, preserved in TRI Reagent (Molecular Research Center, Inc.) and forwarded to Ann Arbor. Three veneroid bivalves were used as outgroups: the marine *Lasaea australis*, and two non-sphaeriid exotic freshwater bivalves collected in Michigan [*Dreissena polymorpha* and *Corbicula leana* (Siripattrawan *et al.*, 2000)]. Table 3-2 summarizes voucher specimen information and GBDB accession numbers for the 11 bivalve mollusk species sequenced in this study.

## **RNA Isolation, RT-PCR, Direct Sequencing and Cloning of PGD cDNA**

Total RNA was prepared from either live or  $-70^{\circ}\text{C}$  frozen tissue. Twenty-thirty mg of mantle tissue (or entire specimens in the case of the smaller species) was processed per individual. The tissue samples were homogenized using QIAshredder centrifuge columns (QIAGEN), processed with TRI Reagent according to the manufacturer's (Molecular Research Center, Inc.) instructions, and the RNA precipitated with the aid of glycogen (Bohringer Mannheim). Total RNA was employed as a template for a 60 minute reverse-transcriptase (RT) reaction ( $42^{\circ}\text{C}$  annealing temperature) utilizing random hexamer primers (Bohringer Mannheim). The RT product then served as a template for the PCR amplification of a 529 nt PGD c-DNA fragment using primers developed by J. Quattro (University of South Carolina): 168F (5'-GARCCNTGYTGYGANTGGGT-3') and 362R (5'-ATRCANCCNCCNCKCCACAT-3'), and *Taq* polymerase (Promega, Buffer a). Primer 168F performed intermittently for the sphaeriid taxa examined, so after generating initial sequences, a sphaeriid-specific primer 178F (5'-GGTGCTGGYCATTGTTGTC-3'), 30 nt downstream from 168F, was designed and used to amplify the target gene fragment from most sphaeriid species.

A touchdown protocol for annealing temperature was followed: the initial temperature of  $64^{\circ}\text{C}$  (40 sec) was decreased by increments of  $2^{\circ}\text{C}$  for the first 7 amplification cycles, then followed by an additional 35 cycles of amplification (40 sec  $94^{\circ}\text{C}$  denaturing, 40 sec  $50^{\circ}\text{C}$  annealing, 1 min  $72^{\circ}\text{C}$  extension). A negative control (no template) was included in each amplification run. Direct sequencing of the amplified cDNA PGD fragment was performed for every species studied. Double-stranded PCR products were isolated on 1% agarose gels, excised under UV light, and purified using a QIAEX II Gel Extraction Kit (QIAGEN). Both strands of amplified products were directly cycle-sequenced using BigDye Terminator Cycle Sequencing Ready Reaction

(Perkin Elmer Applied Biosystems) using the original PCR primers (45°C annealing temperature) and electrophoresed on an ABI 377 automated DNA sequencer.

The target PGD fragment of the seven polyploid sphaeriid species displayed pronounced intra-individual heterozygosity when directly sequenced. In order to accurately sequence individual alleles, these RT-PCR products were cloned into a plasmid vector using the pGEM-T EasyVector System I kit (Promega) which was then used to transform competent bacterial cells (DH5 $\alpha$ ). Ten positive bacterial colonies per individual polyploid sphaeriid clam were harvested and used as templates to amplify and sequence expressed individual PGD alleles. The aim was to establish the predominant allelic variations expressed by each clam, rather than to exhaustively catalogue all such possible alleles.

### **Phylogenetic Analyses**

Resulting chromatograms were edited manually using the Sequence Navigator 1.0.1 (Applied Biosystems). The sequences were compiled with Sequence Monkey 2.8.0 (available from <http://www.monkeysoftwerks.com>), aligned using Clustal\_X (Thompson *et al.*, 1994, 1997; available from <http://ncbi.nlm.nih.gov>.) and the alignment was refined manually where necessary. The possibility of recombination among sequences as well as between the duplicated allele sets was tested using Maximum Chi-Squared (Maynard Smith, 1992; available from [http://www.biols.susx.ac.uk/Biochem/Molbiol/archives/MacOS/Maximum\\_Chi\\_Squared\\_1.0.hqx](http://www.biols.susx.ac.uk/Biochem/Molbiol/archives/MacOS/Maximum_Chi_Squared_1.0.hqx)). All sequences have been deposited in GenBank (for accession numbers see Table 3-2) and the matrices are available from the author. Phylogenetic analyses were conducted using the PAUP\* 4.0b3 (Swofford, 1998) under the maximum parsimony optimality criterion. Three veneroid species, *Lasaea australis*, *Corbicula leana* and *Dreissena polymorpha*, were designated as outgroups.

Analyses were performed as heuristic searches using equal character weighting and random stepwise addition with 100 replications. Inferred sequence gaps were considered as missing data. Branch support levels were estimated with bootstrapping (1000 replications, heuristic searches, 10 random additions each) using PAUP\*, and also with Bremer-Decay Index values (Bremer, 1994) calculated using TreeRot (Sorenson, 1996; available from <http://mightyduck.bu.edu/TreeRot>), which generates a constraint file for PAUP\*.

## Results

### Characteristics of the PGD Allele Sequences

Direct double-stranded sequencing of the target c-DNA PGD fragment yielded unambiguous sequences lacking observable heterozygosity for the three outgroup taxa and for the euperine *Eupera cubensis*. This was not the case for the 7 polyploid sphaeriine taxa in which the expressed allele fragments displayed pronounced intra-individual heterozygosity levels (multiple chromatograph peaks on both strands in discrete nucleotide positions). Cloning of the amplified PGD fragments, followed by sequencing of individual clones, was required to accurately distinguish individual sphaeriine alleles. Of the 70 PGD sequences obtained via cloning (10 clones/polyploid species), 47 represented distinct alleles (Figure 3-2). The full-length sphaeriid ingroup PGD fragment length was 529 nt, 6nt longer than the homologous *Drosophila* PGD sequence (Scott and Lucchesi, 1991). Outgroup sequences differed in length: *Lasaea australis* (529 nt), *Dreissena polymorpha* (526 nt) and *Corbicula leana* (523 nt).

Polyploid sphaeriine c-DNA PGD sequences yielded a striking pattern of expressed intra-individual genotype diversity. Multiple alleles (5-9/individual) were

recovered from each of the 7 typed polyploid sphaeriids (Figure 3-2) and pronounced levels of intra-individual allelic divergence levels were detected in most of these taxa, ranging up to 8.5% in *Sphaerium simile* (Table 3-3). Six of the 47 cloned partial PGD alleles appeared to be non-functional. Frameshift-inducing, single nucleotide deletions were observed in 4 alleles (PadaA4, PadaR2, PdubA6 and PdubA8; Figure 3-2) and two others (SsimA1 and PdubA2; Figure 3-2) contained a premature termination codon. Five putatively recombinant PGD alleles (MsecR1, SstrR1, SstrR2, PadaR1 and PadaR2) were detected. They were characterized by abrupt and dramatic disjunctions in nucleotide sequence similarity along the length of the c-DNA transcript relative to the other PGD alleles expressed by that individual clam (Figure 3-2). These sequence similarity disjunctions were readily detectable by visual alignment (Figure 3-2) and also with the computerized recombination test, Maximum Chi-Squared (Maynard Smith, 1992; results are discussed further below). Slatkin (1994) argued that recombination rates may exceed mutation rates for many nuclear genes, thereby scrambling phylogenetically informative variation. In an effort to minimize such concerns, I removed the 5 putative recombinant PGD alleles from the initial series of phylogenetic analyses.

### **Phylogenetic Analyses of PGD Alleles - Putative Recombinants Excluded**

A total of 46 PGD allele sequences (not including the 5 putative sphaeriine recombinants), acquired from 8 sphaeriids and 3 outgroup taxa, were aligned into a matrix of 529 characters. The matrix contained 341 nucleotide variable positions of which 213 were parsimony-informative. The respective values for ingroup (Sphaeriidae) comparisons were 208 and 81. Heuristic searches of the PGD allelic data set under a parsimony optimality criterion yielded 28 equally parsimonious trees (647 steps, CI = 0.781, RI = 0.841) and a strict consensus is depicted in Figure 3-3.

Robust support for monophyly of the Sphaeriidae and for a sister relationship of *Eupera cubensis* to the polyploid Sphaeriinae are evident in the strict consensus PGD tree topology (Figure 3-3). The cornucopia of polyploid sphaeriine PGD allelic diversity resolved into two clades (Clades A and B in Figure 3-3) and clade-specific prefixes (A, B) were assigned to each allele (Figure 3-2) reflecting its respective phylogenetic placement (Figure 3-3). The two clades were unequally represented in the sample of expressed PGD alleles among and within polyploid species. Clade A alleles were more diverse than the Clade B repertoire in the fraction of the polyploid species they encompassed (7 versus 3), in the number of genotypes recovered (35 versus 7), and in their uncorrected within-clade genetic divergence levels ( $\leq 7.4\%$  versus  $\leq 3.0\%$ ; Table 3-3).

All three *Sphaerium* taxa expressed both Clade A and Clade B alleles. In each of the *Sphaerium* species investigated, a subset of PGD alleles (Clade A) were sister, not to the rest of an individual clam's PGD allelic repertoire, but to Clade A alleles expressed by all of the other 6 polyploid species investigated including species of *Musculium* and *Pisidium*. This result is reflected in the high degree of intra-individual genetic divergence ( $\geq 33$  variable positions; uncorrected sequence divergence of  $\geq 6.2\%$ ) observed in *Sphaerium* PGD allelic complements. Clade B *Sphaerium* PGD alleles display species-level monophyly and their topological inter-relationships (*S. occidentale* (*S. simile*, *S. striatinum*)) are congruent to the reciprocal section of Clade A.

Non-recombinant PGD allelic repertoires of polyploid *Musculium* and *Pisidium* taxa (*M. securis*, *P. dubium*, *P. compressum* and *P. adamsi*) lacked Clade B representatives. *Musculium securis* alleles were recovered as monophyletic and grouped with *Sphaerium* Clade A alleles forming an unresolved polytomy. *Pisidium* taxa formed a paraphyletic assemblage and were sister to *Sphaerium/Musculium* Clade A genotypes. *P. dubium* alleles formed a species-specific clade while *P. compressum* and *P. adamsi* alleles grouped together. Intra-individual allelic diversity in *Musculium* and in all three

*Pisidium* taxa were less than that of the *Sphaerium* taxa, markedly so for *P. dubium* and, especially, *P. compressum* (Table 3-3).

### **Phylogenetic Analyses of PGD Alleles - Putative Recombinants Included**

The 5 putative recombinant (PR) PGD alleles (MsecR1, SstrR1, SstrR2, PadaR1 and PadaR2) were added to the PGD dataset to give a total of 51 genotypes. Phylogenetic analysis yielded 11 equally most parsimonious trees of 725 steps and a strict consensus is shown in Figure 3-4. As in Figure 3-3, sphaeriid and sphaeriine monophyly were well supported. However, the addition of the 5 PR alleles altered details of among-sphaeriine topological relationships and weakened support levels for deeper nodes in the sphaeriine clade (compare bootstrap as well as Decay Index values in Figures 3-3 and 3-4). None of the 5 PR alleles formed monophyletic groups with the remainder of the PGD genotypes recovered from the same *Musculium securis*, *Sphaerium striatinum* and *Pisidium adamsi* individuals (Figure 3-4). One of the PR genotypes, SsstrR2, branched outside both of the 2 major clades (A, B), leading to a trichotomous sphaeriine PGD gene tree topology. Most notably, the PR allele MsecR1, unlike the rest of the PGD alleles expressed by *M. securis*, clustered within Clade B (Figure 3-4). The remaining PR alleles (SstrR1, PadaR1 and PadaR2) were positioned within Clade A, respectively sister to *Sphaerium/Musculium*, to *P. dubium* and to *P. adamsi/compressum* non-recombinant PGD alleles.

## Recombination Tests and Phylogenetic Analyses of Partitioned PGD Alleles

The two putative recombinant *Sphaerium striatinum* alleles, SstrR1 and SstrR2, displayed pronounced segmental sequence similarity to both Clade A and Clade B PGD genotypes expressed by the same individual (Figure 3-2). For these putative recombinants, potential recombination events between two Clade A and Clade B alleles were strongly suggested by the recombination test using Maximum Chi-Squared (Maynard Smith, 1992) with all  $P$  values  $< 0.001$ .  $P$  values for *Musculium securis* allele MsecR1 were also always significant ( $< 0.001$ ) when one of the *M. securis* Clade A alleles along with any one of the *Sphaerium* Clade B PGD genotypes were designated as possible parental sequences. It is therefore easy to envisage their genesis via recombination events involving Clade A and Clade B ancestral alleles. This can be demonstrated by partitioning the PGD data set at various increments along the c-DNA transcript and phylogenetically analyzing the separate sequence segments. Figure 3-5 shows a typical result generated by partitioning the data set at nt position 300. Note that the 2 major sphaeriine clades, A and B, are recovered for both sequence partitions and that putative recombinant alleles MsecR1, SstrR1 and SstrR2 move among the two clades in the different partitions. Although this result strongly supports a recombinant origin for these alleles, it does not rule out the possibility that they are artifacts. Artificial recombinants can be produced by the polymerase chain reaction as a consequence of premature termination and chimeric annealing of the partial PCR products (Bradley and Hillis, 1997). I consider that this process is unlikely to explain the presence of alleles MsecR1, SstrR1 and SstrR2 because they contain multiple substitutions not found in the remainder of the PGD genotypes expressed by the same individual clam (Figure 3-2). It is therefore likely that alleles MsecR1, SstrR1 and SstrR2 represent ancestral recombination events involving Clade A and Clade B allelic precursors.

The two putative recombinant *Pisidium adamsi* alleles, PadaR1 and PadaR2, were inherently enigmatic. Although segments of both displayed pronounced sequence similarity to the other PGD genotypes expressed by the same individual (all Clade A), the remaining segments of the c-DNA transcript did not (Figure 3-2). Where might have these deviant segments originated? Recombination tests (Maynard Smith, 1992) yielded an ambiguous result with the deviant segments showing an association with both Clade B *Sphaerium* and with Clade A *P. dubium* alleles. When phylogenetic analyses were performed with partitioned datasets, both segments of PadaR2 remained within Clade A (Figure 3-5), irrespective of the truncation point utilized. This was a surprising result for the 3' fragment of PadaR2, which differs comprehensively from expressed conspecific Clade A alleles (Figure 3-2) yet remains nested within Clade A sister to its conspecific PR allele PadaR1 (Figure 3-5b). In contrast, partitioning of PadaR1 resulted in the clustering of the 5' 300 nt portion in Clade B (Figure 3-5a). This latter result was interesting because it represented the first appearance of a *Pisidium* PGD segment in sphaeriine Clade B.

## Discussion

A major section of sphaeriid PGD treespace was incongruent with the topological relationships predicted for evolutionarily recent polyploidization (Figure 3-1a). Intra-individual allelic repertoires of the 3 *Sphaerium* taxa investigated did not form species-specific clades. Rather, they were polyphyletic and exhibited cross-species sister relationships consistent with a shared ancestral genome duplication pre-dating their cladogenesis (Figure 3-1b). Placement of the putative recombinant allele MsecR1 in Clade B (Figures 3-4 and 3-5a) was significant in that it revealed a complementary polyphyly in *Musculium securis* which pushes back the inferred genome duplication

event past the common ancestor of these North American *Sphaerium/Musculium* taxa. Although recovered only in recombinant form, the *Musculium* Clade B affinity was strongly supported by both recombination tests and by phylogenetic analyses of partitioned datasets (Figure 3-5a).

The PGD gene tree *Sphaerium/Musculium* sister-relationship (Figures 3-3 and 3-5) is congruent with recent molecular phylogenies based on nuclear ribosomal (Park and Ó Foighil, 2000; Chapter 5) and mitochondrial (Cooley and Ó Foighil, 2000) gene sequences, but not with a recent morphological phylogeny (Dreher-Mansur and Meier-Brook, 2000). Cooley and Ó Foighil (2000) extensively sampled global *Sphaerium/Musculium* taxa, including the 4 exemplar species employed in the present study, and found that these 4 species were inclusive of much of the phylogenetic diversity evident in the *Sphaerium/Musculium* mitochondrial clade. Therefore, it seems probable that the putative ancestral genome duplication event inferred from the PGD tree topologies stems from early in the history of this clade of sequentially brooding sphaeriids (Cooley and Ó Foighil, 2000). It is difficult to place the inferred *Sphaerium/Musculium* ancestral genome duplication event temporally. Sphaeriid fossil relationships are poorly understood, however *Sphaerium* and *Musculium* first appeared in the Cretaceous and Miocene respectively (Keen and Dance, 1969). Although the dataset does not include PGD alleles of *S. corneum*, the only known diploid sphaeriid (Keyl, 1956), incorporation of this species is unlikely to alter the primary conclusion. Irrespective of the placement of *S. corneum* alleles in the PGD tree, it is not possible to establish monophyly for the polyphyletic intraspecific PGD repertoires of *S. striatinum*, *S. simile*, *S. occidentale* and *M. securis* (Figure 3-3).

In contrast to the *Sphaerium/Musculium* genotypes, intra-individual *Pisidium* *dubium* and *P. compressum* PGD repertoires resolved into species-specific clades. This result is inconsistent with a genome duplication event predating cladogenesis of these two polyploid congeners. *Pisidium adamsi* PGD allelic diversity was considerably more

complicated. Most of its alleles were robustly sister to the *P. compressum* clade (Figure 3-3) and this topological relationship (*P. dubium* (*P. adamsi*, *P. compressum*)) was congruent with that obtained in sphaeriid mitochondrial gene trees (Cooley and Ó Foighil, 2000). However, *P. adamsi* also expressed putatively recombinant alleles containing highly divergent segments, one of which (PadaR1) clustered in Clade B in partitioned analyses (Figure 3-5a).

Potential persistence of a fragmentary Clade B allelic motif in *Pisidium adamsi* raises the possibility that the Clade A/B dichotomy may stem back to the common ancestor of all 7 sphaeriine taxa studied. This evolutionary scenario would require secondary loss of Clade B allelic expression in the *Pisidium* taxa through processes of gene silencing and recombination. Such patterns of loss and conversion of duplicated genes are common in polyploid organisms (Wendel, 2000) and there is some evidence that it is ongoing among sphaeriine PGD allelic complements. Six of the 47 expressed PGD alleles recovered appeared to be non-functional (Figure 3-2) and expressed Clade A alleles outnumber Clade B representative in *Sphaerium* species, especially so when the frequency of alleles detected is counted (19 versus 9). Nevertheless, the evidence supporting relic persistence of a Clade B PGD segment in *P. adamsi* allele PadaR1 is far from convincing, especially considering that additional divergent segments in another putative *P. adamsi* recombinant (PadaR2) remain in Clade A when partitioned (Figure 3-5). The provenance(s) of the *P. adamsi* divergent PGD segments remain to be established and discovery of more convincing PGD Clade B alleles in species of *Pisidium* is required to entertain seriously the hypothesis that a shared ancestral genome duplication is common to all three genera.

In addition to providing insights into the temporal interrelationship of genome duplication and speciation events among the study taxa, the present PGD results are also relevant to qualitative inferences into the potential origins of genome duplication in polyploid sphaeriines. As defined by Thompson and Lumaret (1992), genome

duplication may: 1) occur within a species (autopolyploidy); 2) result from hybridization among ancestral species that were partially cross-fertile (segmental allopolyploids); 3) result from hybridization among ancestral species that were almost completely cross-sterile (genomic allopolyploids). The monophyletic PGD allelic complements of *Pisidium dubium* and *P. compressum* are consistent with evolutionarily recent autopolyploidization. In contrast, the polyphyletic PGD profiles of *Sphaerium/Musculium* taxa are indicative of a relatively ancient genomic duplication event that, with the exception of three putatively recombinant *S. striatinum/M. securis* alleles (Figures 3-1 and 3-4), has not been overwritten by subsequent recombination and/or gene conversion. Recombination rates drop rapidly with increasing levels of genetic divergence in organisms with functional mismatch repair systems (Vulic *et al.*, 1999) and the divergent Clade A and Clade B PGD alleles expressed by individual *Sphaerium/Musculium* clams may result in within-clade recombination events being much more common than among clade events. Such a pattern of allelic evolution is consistent with segmental and/or genomic allopolyploidization where the divergent allelic sets are produced by hybridization events among genetically differentiated parental species (Soltis and Soltis, 1993). For instance, genomic allopolyploid lineages contain two complete ancestral parental genomes which exhibit disomic inheritance (Gaut and Doebley, 1997). An allopolyploid origin for the *Sphaerium/Musculium* polyphyletic PGD allelic complement would entail that Clades A and B stem from genetically distinct parental species. These putative parental species are presumably extinct, however the phylogenetic distinctiveness of the euperine *Eupera cubensis* PGD genotype (Figure 3-3) indicates that informative sister alleles are most likely to be found within the Sphaeriinae. One promising place to look for such is among the Gondwanan *Afropisidium* lineages, which were not sampled in this study but have a basal position among the Sphaeriinae in mitochondrial gene trees (Cooley and Ó Foighil, 2000).

Pronounced genome amplification may be prevalent in the cosmopolitan freshwater bivalve family Sphaeriidae (Park, 1992; Baršienė *et al.*, 1996; Burch *et al.*, 1998; Lee, 1999) and ancestral patterns of polyploidization may underlay much of the present diversity of this freshwater clam radiation. The present PGD study represents the first effort to phylogenetically reconstruct this process. Although preliminary, the results of this study indicate that North American *Sphaerium/Musculium* lineages may share ancestral genome duplication events that stem from early in the history of sequentially brooding sphaeriids. In contrast, some of the *Pisidium* species investigated may be the products of evolutionarily recent polyploidization. An in-depth sampling of the Sphaeriidae is required to put these initial results into a comprehensive phylogenetic perspective.

## References

- Baršienė, J., G. Tapia, and D. Barsyte, 1996. Chromosomes of molluscs inhabiting some mountain springs of eastern Spain. *Journal of Molluscan Studies*, 62: 539-543.
- Begun, D. J. and C. F. Aquadro, 1991. Molecular population genetics of the distal portion of the X chromosome in *Drosophila*: evidence for genetic hitchhiking of the yellow-achaete region. *Genetics*, 129: 1147-1158.
- Begun, D. J. and C. F. Aquadro, 1994. Evolutionary inferences from DNA variation at the 6-phosphogluconate dehydrogenase locus in natural populations of *Drosophila*: selection and geographic differentiation. *Genetics*, 136: 155-171.
- Bennett, S. T., A. Y. Kenton, and M. D. Bennett, 1992. Genomic *in situ* hybridization reveals the allopolyploid nature of *Milium montianum* (Gramineae). *Chromosoma*, 101: 420-424.
- Bradley, R. D. and D. M. Hillis, 1997. Recombinant DNA sequences generated by PCR amplification. *Molecular Biology and Evolution*, 14: 592-593.
- Bremer, K., 1994. Branch support and tree stability. *Cladistics*, 10: 295-304.
- Burch, J. B., G-M. Park, and E-Y. Chung, 1998. Michigan's polyploid clams. [Abstract]. *Michigan Academician*, 30: 351-352.
- Campbell, C. S., M. F. Wojciechowski, B. G. Baldwin, L. A. Alice, and M. J. Donoghue, 1997. Persistent nuclear ribosomal DNA sequence polymorphism in the *Amelanchier* Agamic complex (Rosaceae). *Molecular Biology and Evolution*, 14: 81-90.
- Cooley, L. R. and D. Ó Foighil, 2000. Phylogenetic analysis of the Sphaeriidae (Mollusca: Bivalvia) based on partial mitochondrial 16S rDNA gene sequences. *Invertebrate Biology*, 119: 299-308.
- Cronn, R. C., R. L. Small, and J. F. Wendel, 1999. Duplicated genes evolve independently after polyploid formation in cotton. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 14406-14411.
- Dreher-Mansur, C. D. and C. Meier-Brook, 2000. Morphology of *Eupera* Bourguignat 1854, and *Byssanodonta* Orbigny 1846 with contributions to the phylogenetic systematics of Sphaeriidae and Corbiculidae (Bivalvia: Veneroida). *Archiv für Molluskenkunde*, 128: 1-59.
- Dufresne, F. and P. D. N. Herbert, 1994. Hybridization and origins of polyploidy. *Proceedings of the Royal Society of London*, Ser. B. 258: 141-146.

- Eckbald, J. W., N. L. Peterson, K. Ostlie, and A. Tempte, 1977. The morphometry, benthos and sedimentation rates of a floodplain lake in Pool 9 of the upper Mississippi River. *American Midland Naturalist*, 97: 433-443.
- Eudeline, B., S. K. Allen, and X. M. Guo, 2000. Optimization of tetraploid induction in Pacific oysters, *Crassostrea gigas*, using first polar body as a natural indicator. *Aquaculture*, 187: 73-84.
- Fuertes Aguilar, J., J. A. Rosselló, and G. Nieto Feliner, 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). *Molecular Ecology*, 8: 1341-1346.
- Gaut, B. S. and J. F. Doebley, 1997. DNA sequence evidence for the segmental allotetraploid origin of maize. *Proceedings of the National Academy of Sciences of the United States of America*, 94: 6809-6814.
- Hillis, D. M., C. Moritz, C. A. Porter, and R. J. Baker, 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Science*, 251: 308-251.
- Hornbach, D. J., T. E. Wissing, and A. J. Burky, 1984. Energy budget for a stream population of the freshwater clam, *Sphaerium striatinum* Lamarck (Bivalvia: Pisidiidae). *Canadian Journal of Zoology*, 62: 2410-2417.
- Hugall, A., J. Stanton, and C. Moritz, 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution*, 16: 157-164.
- Keen, M. and P. Dance, 1969. Family Pisidiidae. In: *Treatise on Invertebrate Paleontology*. Part. N, Vol. 2, Mollusca 6, Bivalvia. (R. C. Moore, ed.), pp. N669-N670. Geological Society of America and University of Kansas Press, Lawrence, Kansas.
- Kenton, A., A. S. Parokonny, Y. Y. Gleba, and M. D. Bennett, 1993. Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. *Molecular and General Genetics*, 240: 159-169.
- Keyl, H. G., 1956. Beobachtungen über die meiose der muschel *Sphaerium corneum*. *Chromosoma*, 8: 12-17.
- Lee, T., 1999. Polyploidy and meiosis in the freshwater clam *Sphaerium striatinum* (Lamarck) and chromosome numbers in the Sphaeriidae (Bivalvia, Veneroida). *Cytologia*, 64: 247-252.
- Leitch, I. J. and M. D. Bennett, 1997. Polyploidy in angiosperms. *Trends in Plant Science*, 2: 470-476.

- Lopez, G. R. and I. J. Holopainen, 1987. Interstitial suspension-feeding by *Pisidium* spp. (Pisidiidae: Bivalvia): a new guild in the lentic benthos? *American Malacological Bulletin*, 5: 21-30.
- Maynard Smith, J., 1992. Analyzing the mosaic structure of genes. *Journal of Molecular Evolution*, 34: 126-129.
- Muller, H. J., 1925. Why polyploidy is rarer in animals than in plants. *American Naturalist*, 59: 346-353.
- Nakamura, H. K., 1985. A review of molluscan cytogenetic information based on the CISMOCH-Computerized Index System for Molluscan Chromosomes. Bivalvia, Polyplacophora and Cephalopoda. *Venus*, 44: 193-225.
- Ó Foighil, D. and C. Thiriou-Quévieux, 1999. Sympatric Australian *Lasaea* species (Mollusca: Bivalvia) differ in their ploidy levels, reproductive modes and developmental modes. *Zoological Journal of the Linnean Society*, 127: 477-494.
- Okamoto, A. and B. Arimoto, 1986. Chromosomes of *Corbicula japonica*, *C. sandai* and *C. (Corbiculina) leana* (Bivalvia: Corbiculidae). *Venus*, 45: 194-202.
- Orr, H. A., 1990. "Why polyploidy is rare in animals than in plants" revisited. *American Naturalist*, 136: 759-770.
- Park, J-C., 1992. *Taxonomic Study of Musculium japonicum and Pisidium (Neopisidium) coreanum (n. sp.) of Sphaeriidae (Pelecypoda; Veneroida) in Korea*. Master's thesis. Kangwon National University, Korea. 49 pp.
- Park, J-K. and D. Ó Foighil, 2000. Sphaeriid and corbiculid clams represent separated heterodont bivalve radiations into freshwater environments. *Molecular Phylogenetics and Evolution*, 14: 75-88.
- Roelofs, D., J. van Velzen, P. Kuperus, and K. Bachmann, 1997. Molecular evidence for an extinct parent of the tetraploid species *Microseris acuminata* and *M. campestris* (Asteraceae, Lactuceae). *Molecular Ecology*, 6: 641-649.
- Sang, T., D. J. Crawford, and T. F. Stuessy, 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences of the United States of America*, 92: 6813-6817.
- Scott, M. J. and J. C. Lucchesi, 1991. Structure and expression of the *Drosophila melanogaster* gene encoding 6-phosphogluconate dehydrogenase. *Gene*, 109: 177-183.

- Siripattawan, S., J-K. Park, and D. Ó Foighil, 2000. Two lineages of the introduced Asian freshwater clam *Corbicula* occur in North America. *Journal of Molluscan Studies*, 66: 423-429.
- Slatkin, M., 1994. Cladistic analysis of DNA sequence data from subdivided populations. In: *Ecological Genetics*. (L. A. Real, ed.), pp. 18-34, Princeton University Press, Princeton, New Jersey.
- Soltis, D. E. and P. S. Soltis, 1993. Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences*, 12: 243-273.
- Soltis, D. E., and P. S. Soltis, 1999. Polyploidy: recurrent formation and genome evolution. *Trends in Ecology and Evolution*, 14: 348-352.
- Song, K., P. Lu, K. Tang, and T. C. Osborn, 1995. Rapid change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. *Proceedings of the National Academy of Sciences of the United States of America*, 92: 7719-7723.
- Sorenson, M. D., 1999. *TreeRot*, v. 2. Boston University, Boston, Massachusetts. Freeware and documentation available from <http://mightyduck.bu.edu/TreeRot>.
- Swofford, D. L., 1998. *PAUP 4.0.b3: Phylogenetic Analysis Using Parsimony*, version 4.0. Sinauer Associates, Inc., Sunderland, Massachusetts.
- Thompson, J. D. and R. Lumaret, 1992. The evolutionary dynamics of polyploid plants: origins, establishment and persistence. *Trends in Ecology and Evolution*, 7: 302-307.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22: 4673-4680.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, 1997. The CLUSTAL\_X window interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25: 4876-4882. Freeware available from <http://ncbi.nlm.nih.gov>.
- van Houten, W. H. J., N. Scarlett, and K. Bachmann, 1993. Nuclear DNA markers of the Australian tetraploid *Microseris scapigera* and its North American diploid relatives. *Theoretical and Applied Genetics*, 87: 498-505.
- Vulic, M., R. E. Lenski, and M. Radman, 1999. Mutation, recombination, and incipient speciation of bacteria in the laboratory. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 7348-7351.
- Wendel, J. F., 2000. Genome evolution in polyploids. *Plant Molecular Biology*, 42: 225-249.

- Wendel, J. F., A. Schnabel, and T. Seelanan, 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). *Proceedings of the National Academy of Sciences of the United States of America*, 92: 280-284.
- Widmer, A. and M. Baltisberger, 1999. Molecular evidence for allopolyploid speciation and a single origin of the narrow endemic *Draba ladina* (Brassicaceae). *American Journal of Botany*, 86: 1282-1289.

**Table 3-1.** Mitotic chromosome numbers and locality data for the taxa used in this study.

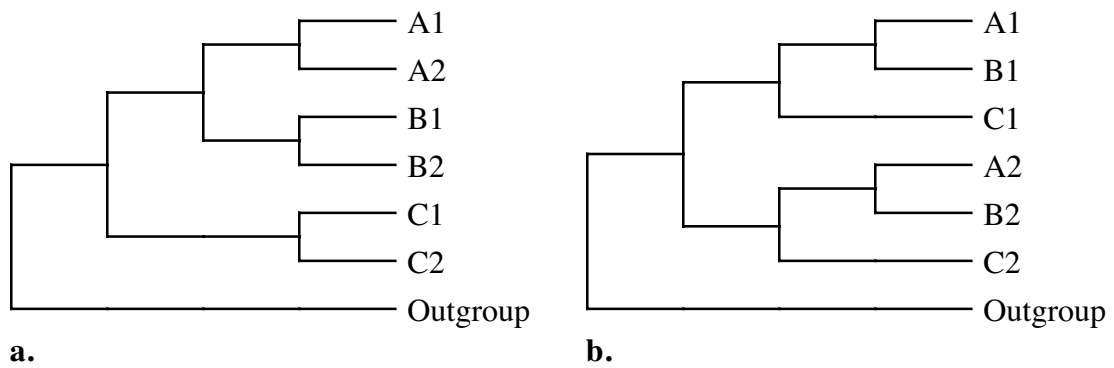
Taxonomic Position	Mitotic Chromosome No.	Collection Locality
Class Bivalvia		
Subclass Heterodonta		
Order Veneroida		
Family Sphaeriidae		
<i>Eupera cubensis</i> (Prime, 1865)	?	Havana, Cuba
<i>Musculium securis</i> (Prime, 1852)	247 (Burch <i>et al.</i> , 1998)	Mich., USA
<i>Sphaerium occidentale</i> (Lewis, 1856)	209 (Burch <i>et al.</i> , 1998)	Mich., USA
<i>Sphaerium simile</i> (Say, 1822)	> 100	Mich., USA
<i>Sphaerium striatinum</i> (Lamarck, 1818)	152 (Lee, 1999)	Mich., USA
<i>Pisidium compressum</i> Prime, 1852	> 100	Mich., USA
<i>Pisidium dubium</i> (Say, 1817)	> 200	Mich., USA
<i>Pisidium adamsi</i> Stimpson, 1851	> 100	Mich., USA
Family Lasaeidae		
<i>Lasaea australis</i> (Lamarck, 1818)		Tasmania, Australia
Family Corbiculidae		
<i>Corbicula leana</i> Prime, 1864		Mich., USA
Family Dreissenidae		
<i>Dreissena polymorpha</i> (Pallas, 1771)		Mich., USA

**Table 3-2.** Voucher specimen information (Mollusk Division Catalog Number, University of Michigan, Museum of Zoology), and GenBank accession number for the taxa used in this study.

Taxonomic Position	UMMZ	GBDB
	Catalog No.	Accession No.
Class Bivalvia		
Subclass Heterodonta		
Order Veneroida		
Family Sphaeriidae		
<i>Eupera cubensis</i> (Prime, 1865)	266709	AF345447
<i>Musculium securis</i> (Prime, 1852)	266710	AF345448-56
<i>Sphaerium occidentale</i> (Lewis, 1856)	266711	AF345457-61
<i>Sphaerium simile</i> (Say, 1822)	266712	AF345462-68
<i>Sphaerium striatinum</i> (Lamarck, 1818)	266713	AF345469-75
<i>Pisidium compressum</i> Prime, 1852	266714	AF345476-80
<i>Pisidium dubium</i> (Say, 1817)	266715	AF345481-88
<i>Pisidium adamsi</i> Stimpson, 1851	266716	AF345489-94
Family Lasaeidae		
<i>Lasaea australis</i> (Lamarck, 1818)	266717	AF345495
Family Corbiculidae		
<i>Corbicula leana</i> Prime, 1864	266693	AF345496
Family Dreissenidae		
<i>Dreissena polymorpha</i> (Pallas, 1771)	266719	AF345497

**Table 3-3.** Sequence divergence of PGD alleles expressed by a single individual clam of polyploid sphaeriid species and by allele groups.

Species	# of alleles obtained	Uncorrected pairwise distance		
		Maximum	Minimum	Average
<i>M. securis</i>	9	0.0794	0.0076	0.0275
<i>S. occidentale</i>	5	0.0700	0.0019	0.0412
<i>S. simile</i>	7	0.0851	0.0019	0.0423
<i>S. striatinum</i>	7	0.0813	0.0038	0.0486
<i>P. compressum</i>	5	0.0076	0.0019	0.0045
<i>P. dubium</i>	8	0.0227	0.0019	0.0136
<i>P. adamsi</i>	6	0.0606	0.0095	0.0369
Subtotals for cloned allele clades				
Clade A	35	0.0738	0.0019	0.0445
Clade B	7	0.0302	0.0038	0.0173



**Figure 3-1.** Predicted phylogenetic trees for 6-Phosphogluconate Dehydrogenase allelic variation among polyploid sphaeriid clam species (A, B and C) if duplicated alleles (1 and 2) have maintained evolutionary independence and expression. **a.** alleles from each species will be monophyletic if post-speciation polyploidization occurred; **b.** each species alleles will be polyphyletic if polyploidization preceded speciation.

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**Figure 3-2.** Variable nucleotide sites for PGD alleles of polyploid sphaeriids. To enhance legibility, alleles and/or allele fragments are color coded to reflect their positions in phylogenetic analyses (Figures 3-5). Clade A alleles are colored in blue, Clade B alleles in red, and putative recombinants in more than one color. A dash (-) indicates a single inferred nucleotide deletion and inferred non-sense mutations are underlined.

<i>M. securis</i>	
Variable nucleotide position	
	111111 1111111112 222222222 2222233333 3334444444 5
	1133555677 7889001134 4557778992 2223345556 6788901223 5571244688 1
Allele	0218256413 7075360202 3472384090 3450800342 8289881267 0501918624 7
MsecA1	CACCCTCGCA GAAACGGCGT TAAGTATGCA TGGAAGAAGG AGCAATAATG AGGACATCAT T
MsecA2	.....C .....A..A.....T.....T.....
MsecA3	.....C .....A.CC..G.....T..C.....T.....
MsecA4	.....C .....A.....T...T.....TGC...
MsecA5	.....C .....A.....G.....T.....TG.T..
MsecA6	.G...T...C...C..A...A...T.....T.....
MsecA7	.....T...C..G...CA.....T.....T...TT...C..
MsecA8	.....TA...G.....C.....A.....AT.....TT...T..
MsecR1	T.TTAC..TG AT.GTAATAC C.GA.G.AT. .A.G.GCAA G.TGG.CGCA .AA.T.....C

<i>S. occidentale</i>	
Variable nucleotide position	
	111111 1122222223 3333333333 34444455
	1378001113 5900356790 0122356777 80335612
Allele	0110360240 9908838181 4126754179 53697376
SoccA1	CCCACGGCGG GCCAAAATAC GAATGGTGTG TGTAATG
SoccA2	.....A. ...G.....
SoccA3	.....A. A..G.....
SoccB1	TTTTTAATAA .T.GGCGCGT TCGCATCTCA CAAGTT.C
SoccB2	TTTTTAATAA .TTGGCGCGT TCGCATCTCA CAAGTTCC

<i>S. simile</i>	
Variable nucleotide position	
	111111111 1112222222 2223333333 3333444444 55
	1112355778 9001122356 7890355566 7790123567 7789023358 02
Allele	0568128130 5360712017 2396834528 1681167540 6981376979 56
SsimA1	CACTCCACAA ACGGACGGAA GCCGAAGTGA TTACATGGCG GGATGCTAAA AG
SsimA2	TGT.T.T... ..G.A... ..A.....C.....T..G.....
SsimA3	.G.C..T... G...G.A... ..A.....T.....G.A
SsimA4	.G...T... ..G.A... ..A.....T.....
SsimA5	.G...T... ..G.A... ..A..C.....T.....
SsimB1	TGT.TTTTGT .TAAG.AAGT ATTAGCA.AG C.GTCCAATA AA.GAAAGT. GC
SsimB2	TGT.TTTTGT .TAAGTAAG. A.TAGCA.AG C.GTCCAATA AA.GAAAGT. GC

Figure 3-2.

<i>S. striatinum</i>	
Variable nucleotide position	
	1 1111111112 2222222233 3333333333 3444444455 5
	1134577890 0013557793 4556678901 2233567779 9033568800 2
	0612213013 6800012398 2342812811 2637530691 8369731845 6
SstrA1	CCCTCCAATC GTGGAAGTCA AAGGATTACA ATCGGAGGGT AGTAAAAAAA A
SstrA2	.....G. ....G...
SstrR1	TTT.TTGT.T ACAAGGA... ..T.....
SstrR2	.....C.. .CAAGC.GTC GC.AAG.... ..AGTTG..G C
SstrB1	TTT.TTGT.T A.AA.GA.TG .CAAGCCGTC GC.AAGAAAG GAAGTT...G C
SstrB2	TTTATTGT.T A.AA.GA.TG .CAAGC.GTC GC.AAGAAAG .AAGTT...G C
SstrB3	TTT.TTGT.T A.AA.GA.TG GCAAGC.GTC GC.AAGAAAG .AAGTT..GG C

<i>P. compressum</i>	
	113
	39774
	963389
PcomA1	AAGTGC
PcomA2	..A.A.
PcomA3	..A...
PcomA4	GGA...
PcomA5	..AC.A

<i>P. dubium</i>	
Variable nucleotide position	
	11 1111222222 333334444
	122444801 1377012246 056781678
Allele	7319249460 2237970517 666741966
PdubA1	CTAGTTAAAA TTTAATAGAA TCGTTAGAC
PdubA2	TC..C.GGGG CC.T....G. ....G...
PdubA3	T..A...GGG C.C..... .T.....
PdubA4	T....C.GGG C.....
PdubA5	T....C.GGG C.....C....
PdubA6	T.....GGG C....G....-A..AGT
PdubA7	T.....GGG C....G... C.....T
PdubA8	T.G....GGG C...G...-G.....T

<i>P. adamsi</i>	
Variable nucleotide position	
	11 1111111222 2222333333 3333333444 4444444444 55
	1233577901 1356889135 7899011233 4455799001 1234455668 02
Allele	0214228862 3095479485 1607134504 0145957190 5863517354 66
PadaA1	CACGGTGGGC AGGCTTCTGT TAAATATAGA TGCAACTATG TTTAATGCTT CC
PadaA2	.....T.....C.....G.A.....C..
PadaA3	...A.....AT.....C.G.....A.....
PadaA4	.....-.....T.....C.....A..A.....C..G.....
PadaR1	T.T.C.AAAT GA.TCCTCA..G..CG.GAT C.TGGAAGCA.....
PadaR2	.G.....T.C...C..G...GAT CATGGAAGCA .CA.CCCA-. TT

**Figure 3-2. Continued.**

**Figure 3-3.** Phylogenetic analysis of sphaeriid non-recombinant PGD alleles (5 putative sphaeriine recombinants were excluded). A strict consensus was recovered from 28 equally parsimonious trees (L = 647; CI = 0.781; RI = 0.841) based on heuristic analysis of 46 PGD allele sequences. Three veneroid species, *Lasaea australis*, *Corbicula leana* and *Dreissena polymorpha*, 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.





**Figure 3-4.** Phylogenetic analysis of sphaeriid PGD alleles including 5 putative sphaeriine recombinants. A strict consensus was recovered from 11 equally parsimonious trees ( $L = 725$ ;  $CI = 0.719$ ;  $RI = 0.795$ ) based on heuristic analysis of 51 PGD allele sequences. Three veneroid species, *Lasaea australis*, *Corbicula leana* and *Dreissena polymorpha*, 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.

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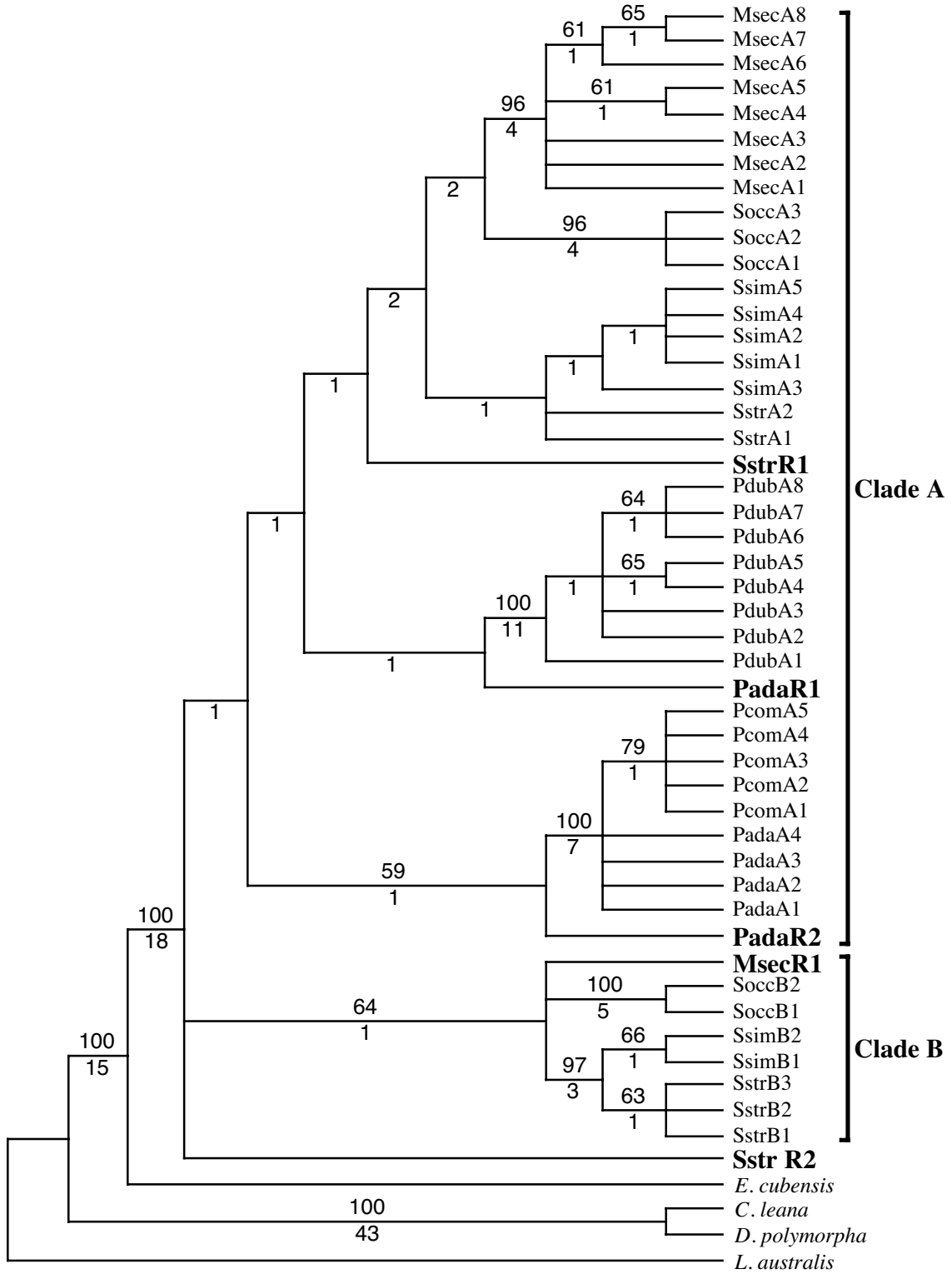


Figure 3-4.

**Figure 3-5.** Phylogenetic analyses of partitioned PGD allele sequences. **a**, strict consensus of 18 equally parsimonious trees ( $L = 375$ ;  $CI = 0.651$ ;  $RI = 0.813$ ) obtained from the heuristic analysis of 5' 300 nt portion of 51 PGD allele sequences; **b**, strict consensus of 231 equally parsimonious trees ( $L = 332$ ;  $CI = 0.669$ ;  $RI = 0.814$ ) obtained from the heuristic analysis of 3' 229 nt portion of 51 PGD allele sequences. Numbers above the branches represent bootstrap values, and numbers below indicate decay index values.

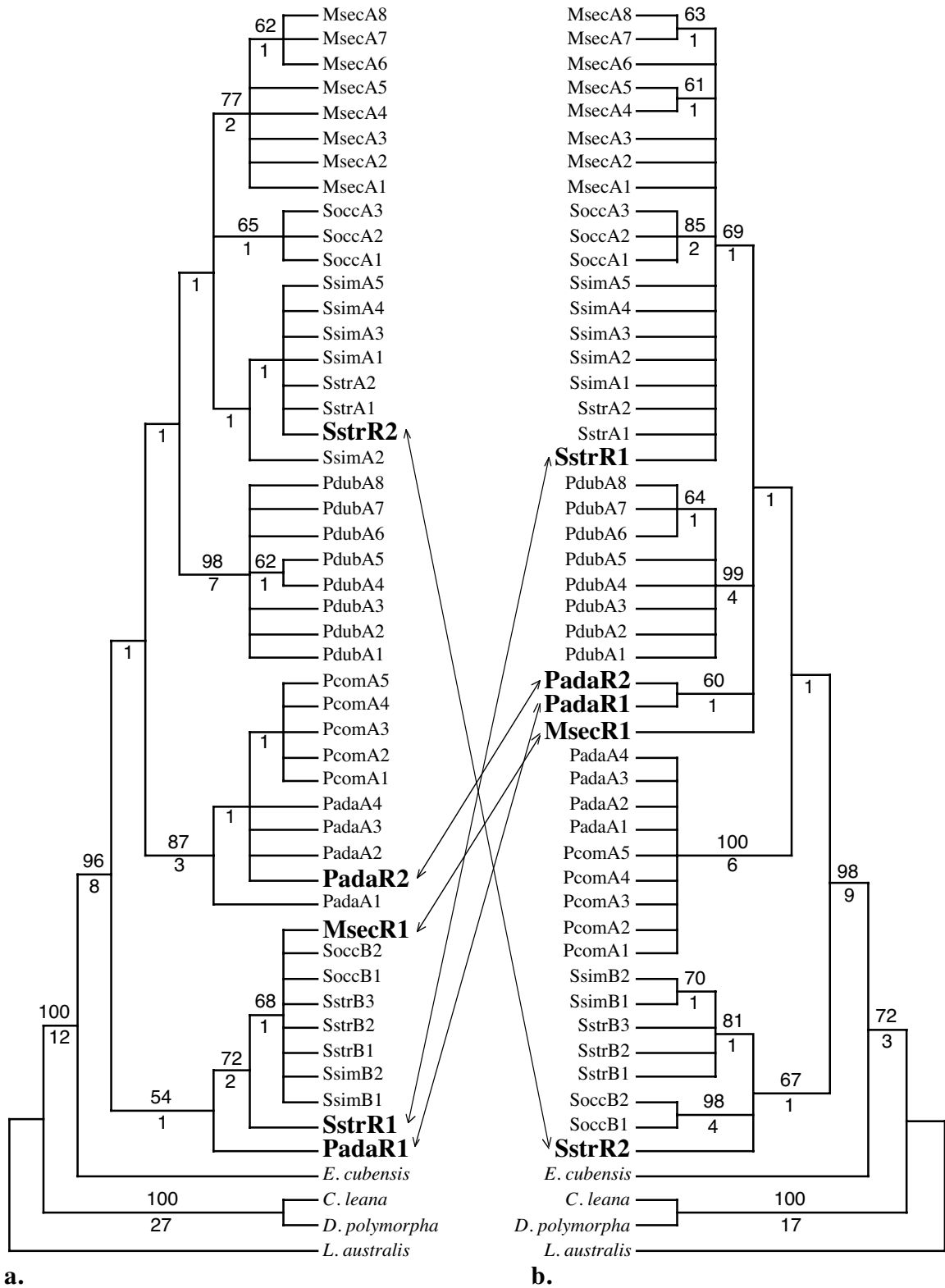


Figure 3-5.