Empirical estimates of reproductive isolation among the *Physa* species of South Carolina (Gastropoda: Pulmonata: Basommatophora)

Robert T. Dillon, Jr.
Department of Biology
College of Charleston
Charleston, SC 29424 USA
dillonr@cofc.edu

ABSTRACT

Previously published mtDNA sequence data have suggested that an undescribed species of *Physa* ("Species A") may inhabit the swamps and ditches in the southeastern Atlantic coastal plain. These snails are characterized by slender shells and dark bodies, but are otherwise similar to the more widely distributed *P. pomilia*. Mate choice tests revealed significant sexual isolation between Species A and *P. pomilia*, with homogametic pairings of *P. pomilia* five times more frequent than heterogametic. A set of no-choice outcross experiments yielded only self-fertilized progeny from the Species A parent and reproductive failure from the *pomilia* parent, suggesting complete Species A x *pomilia* hybrid inviability. The third species of *Physa* inhabiting South Carolina, *P. acuta*, is more genetically similar to Species A but bears a distinctive penial anatomy. Mate choice tests uncovered no evidence of sexual isolation between Species A and *P. acuta*, and hybridization occurred readily, with some reduction in parental fecundity but normal F1 viability. Species A × *acuta* F1 hybrids appear, however, to be 100% sterile. Thus, the relationship between the degree of reproductive isolation and genetic divergence seems to be stronger than that between reproductive isolation and penial anatomy in the physid snails of South Carolina. *Physa* Species A warrants formal description.

Additional keywords: Speciation, *Physella*, *Physa acuta*, *Physa pomilia*, mate choice, sexual isolation, hybridization, allozyme electrophoresis

INTRODUCTION

In recent years, a great deal of interest has focused on the evolutionary biology of freshwater pulmonate snails in the family Physidae (Tsitrone et al., 2003; Bousset et al., 2004; Henry et al., 2005; 2006; Escobar et al., 2007). Their great reproductive plasticity, which includes selfing, mixed-mating, and outcrossing in either or both sexual roles, together with their ease of culture and the availability of genetic markers, has made physid snails a favorite model for the study of sex allocation generally (Dillon and Wethington, 1992; Wethington and Dillon, 1991; 1993; 1996; 1997). But despite great advances in our understanding of broad aspects of their reproductive biology, progress in disentangling the complex evolutionary relationships within the family Physidae has been slow.

The classification system of George Te (1978; 1980) recognized about 40 species and subspecies of physids in North America, arranged into genera and subgenera by penial anatomy. Within the group of nominal species bearing the penial complex Te characterized as "type-b," however, Dillon and Wethington (2006a) reported no reproductive isolation among *P. gyrina* (Say, 1821), and five other more recently described species: *P. ancillaria* (Say, 1825), *P. aurea* (Lea, 1838), *P. microstriata* (Chamberlain and Berry, 1930), *P. parkeri* (Currier in DeCamp, 1881), and *P. utahensis* (Clench, 1925). The addition of *P. sayi* (Tappan, 1838) to the list of type-b synonyms of *P. gyrina* was suggested by the survey of genetic variation at allozyme-encoding loci offered by Dillon and Wethington (2006b).

In the group of physids bearing Te's "penial complex type-c," Dillon et al. (2002) could find no reproductive isolation among *P. integra* (Haldeman 1841) from the American northeast, *P. heterostropha* (Say, 1817) from the American southeast, or the cosmopolitan *P. acuta* (Draparnaud, 1805), described from Europe prior to any American species of physid. *Physa cubensis* (Pfeiffer, 1839), from the Caribbean, and *P. virgata* (Gould, 1855), from the American West, have also recently been synonymized under *P. acuta* (Paraense & Pointier, 2003; Dillon et al., 2005). Reproductive isolation is complete, however, between physids bearing type-b and type-c penial complexes (Dillon et al., 2004).

Te also recognized a group with penial anatomy intermediate between type-b and type-c. These "type-bc" species included *P. hendersoni* (Clench, 1925), originally described as a subspecies of *P. pomilia* (Conrad, 1834). But since Te's observations suggested to him that *P. pomilia* bore type-c penial morphology, he lowered *pomilia* to subspecific status under *P. heterostropha* and

raised *hendersoni* to the rank of species. More thorough observations and experiments have confirmed, however, that topotypic *P. pomilia* bear penial anatomy type-bc, and that they are not reproductively isolated from *P. hendersoni*, a junior synonym (Dillon et al., 2007).

Recently a new classification has been proposed synthesizing laboratory experiments on reproductive isolation together with mtDNA sequence divergence and morphological observations (Wethington, 2004; Wethington and Lydeard, 2007). This classification recognizes approximately 12 North American species and documents a loose correspondence between mtDNA sequence phylogroups and Te's penial morphologies as outlined above.

In addition, the sequence data of Wethington and Lydeard suggests that a previously unrecognized species of *Physa*, characterized by a dark body and elongated shell, might inhabit the swamps and ditches of the southeastern coastal plain. This species, bearing the type-bc penial anatomy of *P. pomilia* but genetically more similar to *P. acuta*, was referred to as "*Physa* Species A". The purpose of the present paper is to report the results of experiments designed to test for reproductive isolation between *Physa* Species A and populations of the two other physids occurring in South Carolina, *P. acuta* (type-c) and *P. pomilia* (type-bc).

The origin and evolution of reproductive isolation has been the subject of intense interest since the early twentieth-century birth of the Modern Synthesis (Mayr, 1942; 1963). The barriers that may evolve between a pair of populations are conventionally divided into prezygotic components (such as sexual isolation) and postzygotic components (such as hybrid inviability or sterility). The former is typically assessed using mate choice tests (Bateson, 1983) and the latter by no-choice breeding experiments (Coyne and Orr, 2004). Here we report the results of both mate choice and no-choice breeding experiments between a reference population of *Physa* Species A from South Carolina and *P. acuta*, then (separately) *Physa* Species A and *P. pomilia*.

MATERIALS AND METHODS

The *Physa acuta* population used to found "line A" for these experiments inhabits the main pond at Charles Towne Landing State Park, west of the Ashley River, within the city limits of Charleston, SC (32.8062° N, 79.9862° W). Snails of this population are not reproductively isolated from *P. acuta* sampled near the type locality for the species in France (Dillon et al., 2002). The *Physa pomilia* population used here to found "line H" was collected from the type locality for *Physa pomilia hendersoni* (Clench, 1925): the Combahee River at the US 21/17A bridge, 1 km E of Yemassee, Hampton County, SC (32.7060° N, 80.8281° W). Dillon et al. (2007) reported no reproductive isolation between this population and snails sampled from Conrad's (1834) type locality for *Physa pomilia* sensu stricto in Alabama. The reference population of *Physa* Species A used to found line "S" was collected from the spring by Huger Creek at Huger Landing, 4 km N of Huger, Berkeley County, South Carolina (33.1305°N; 79.8111°W).

All snails were cultured in transparent polyethylene 10 ounce drinking cups filled with approximately 210 ml of aerated, filtered pond water and covered with a 95 x 15 mm polystyrene Petri dish lid. They were fed O.S.I. Spirulina Aquarium Flake Food, sold in pet stores primarily as a diet for herbivorous aquarium fishes. All experiments took place at room temperature, approximately 23°C. I initially isolated ten wild-collected snails from each study population in separate cups, collected egg masses with weekly water change, and reared the offspring to 2 mm shell length, approximately 3 weeks post-hatching (well in advance of maturity). These three sets of wild-collected but laboratory born sibships were designated A1 through A10, S1 through S10, and H1 through H10. From these sibships were drawn isolates for the mate choice tests and pairs of parents for the study of postzygotic reproductive isolation.

For mate choice tests, large samples of juvenile snails from all three populations were reared to maturity over the course of 8–10 weeks isolated in individual cups, with weekly feeding and water change. Two experiments were performed: one comparing Species A to *P. acuta* and the other comparing Species A to *P. pomilia*. Each experiment was composed of three trials, each trial involving 10 adult snails from one population and ten adult snails from a second, all approximately matching in their shell sizes. Snails were blotted dry and marked with a small dab of fingernail polish according to their population of origin. Then the 20 individuals were simultaneously introduced into a 2 liter glass beaker (filled with 1,400 ml of filtered, aerated pond water) and placed on a glass table to facilitate observation.

Mating activity was monitored for 6 hours. When a snail first successfully copulated as male (defined as the complete insertion of its penis into the gonopore of a partner) it was removed from the beaker, its shell marked with a dot of white correction fluid, and returned. Each individual was often involved in many matings over the 6 hours of observation, both in the male and in the female role, but only its first successful copulation in the male role was recorded. This was an arbitrary decision on my part (since both copulants in a pair might mate in either role, and the result is not a "choice" but rather the outcome of a contest), but necessary nevertheless to prevent double-counting. Note that this design yields a slight bias toward heterogametic pairings, not 1:1 but rather 9:10.

Each trial involved 20 fresh snails, entirely unmated. Three such trials were performed testing for sexual isolation between Species A and *acuta* (the SA experiment) and three additional trials performed testing for sexual isolation between Species A and *pomilia* (the SH experiment), pooling results within experiment to yield a maximum of 60 observations in each case. Chi-square statistics were calculated from the pair of 2 x 2 contingency tables that resulted, normalized by 4/N, as a measure of sexual isolation (Gilbert and Starmer, 1985).

For no-choice tests of postzygotic reproductive isolation, three sets of incross control cups were established using pairs of unrelated parents drawn from the ten sibships within each of the populations (S, H, and A), as for example S1×S2, S2×S3, . . . , S10×S1. Two sets of outcross experimental cups were also established with 10 pairs of snails across populations, the SA cross (S×A1, S×A2, . . . , S×A10) and the SH cross (S×H1, S×H2, . . . S×H10). Each pair of parents received a water change and fresh food every 7 days, at which time the sides of the cup were inspected for egg masses. (Note that any egg mass might result from outcrossing, or be the product of self-fertilization by either parent.) If egg masses were present, all embryos were counted and adults transferred to a fresh cup. Eggs were monitored until hatching (generally about 2 weeks) and all viable, crawling F1 juveniles counted. Observation was terminated upon the death of either parent in a pair.

Crosses were initiated with pairs of snails aged one week post hatch. Then any difference in the central tendency of age at first reproduction (in weeks post hatch) between the 10 outcross pairs and the combination of both sets of 10 corresponding control pairs was tested by calculating a combined (30 pair) median and comparing counts above and below that median using Fisher's exact tests.

For statistical analysis of fecundity and F1 viability, week 1 was established separately for each set of 10 pairs as the first week in which eggs were laid by 3 or more pairs of parents. Embryos and viable hatchlings were subsequently counted for 10 weeks. I then averaged the embryo production of each pair of parents across its lifetime, ignoring any leading (pre-maturity) zeros and any postmortem zeros, while including as 0 any failure to reproduce by viable, mature pairs. So, for example, if one parent in a pair of snails died at week 6, leaving a record of 0, 0, 40, 0, 50 embryos for the pair, their mean fecundity would be 90/3 = 30 embryos per week. A Kruskal-Wallis nonparametric ANOVA was used to test whether any significant difference existed in the central tendency of weekly mean fecundity of either set of 10 outcross pairs (SH or SA) and the 2 corresponding sets of 10 control pairs.

Similarly, I averaged the counts of F1 hatchlings within pairs across weeks, ignoring zeros not corresponding to embryo production, and divided each pair mean by its mean embryo production to obtain pair mean F1 viability. If 35 + 45 hatchlings were recovered from the example pair of snails above, their mean F1 viability would be (35/40 + 45/50)/2 = 88.9%. A second Kruskal-Wallis nonparametric ANOVA was used to test whether any significant difference existed in the central tendency of weekly mean F1 viability posted by either set of 10 outcross pairs and its 2 corresponding sets of 10 control pairs.

To assess the fertility of putative hybrid offspring, F1 hatchlings (from both experimental sets and all three control sets) were reared from each of 3 separate unrelated pairs to size 2 mm. These were crossed in time series: 1 early pair from eggs laid around week 1, 1 middle pair produced around week 5, and 1 late pair produced around week 10, to yield 9 F1 pairs. So if the putative hybrid progeny were reared from pairs S×A1, S×A2, and S×A3, for example, they were crossed as SA1×SA2 early, SA2×SA3 early, SA3×SA1 early, SA1×SA2 middle, SA2×SA3 middle, . . . , SA3×SA1 late. Nine crosses were likewise constituted for corresponding controls S and A, and the total of 3 × 9 = 27 crosses of F1 snails reared to adulthood for each experiment, with weekly feeding and water change. An identical set of 27 cups was established to evaluate hybrid fertility in the SH experiment. I recorded the dates at which embryos and viable F2 hatchlings were produced by each pair.

A larger sample of F1 progeny from 3 outcross pairs from both the SA and SH experiments were reared to 4–5 mm shell length, at which time they were frozen in 100 µl of tissue buffer for analysis by allozyme electrophoresis. We have identified 12 enzyme-encoding loci at which allozyme variation is interpretable as the product of codominant alleles segregating in Mendelian fashion (Dillon and Wethington, 1994). These are aconitase (Acon), esterases (three loci: Est1, Est3, Est6), glucose phosphate isomerase (Gpi), isocitrate dehydrogenase (two loci: Isdh1 and Isdh2), leucine aminopeptidase (Lap), mannose phosphate isomerase (Mpi), phosphoglucomutase (two loci: Pgm1 and Pgm2), and 6-phosphogluconate dehydrogenase (6pgd). We used horizontal starch gel electrophoresis in an aminopropylmorpholine pH 6 buffer system to resolve allozyme variation at the Gpi, Isdh, and 6pgd loci, a Tris-Citrate pH6 buffer system for Acon, Mpi, and Pgm, and a TEB8 system for 6pgd, Lap, and Est. Details regarding our electrophoretic methods, including a description of our equipment and recipes for stains and buffers, have been previously published (Dillon, 1992; Dillon and Wethington, 1995).

The set of no-choice mating experiments described above were conducted simultaneously with those of Dillon et al. (2007), using identical techniques. The data reported here on the reproductive performance of the A and H incross control lines have been published previously, although the SA and SH experimental results, as well as the S incross control, are original to the present investigation.

RESULTS

The SA mate choice experiments did not reveal any evidence of sexual isolation between Species A and *P. acuta* (Table 1, upper). A total of 49 copulations were observed (of a possible 60 total), apparently without regard to species (normalized $\chi^2 = 0.82$, $p = 0.37$). The SH experiments did, however, suggest prezygotic reproductive isolation between Species A and *P. pomilia* (Table 1, lower). The 38 copulations observed in the SH mate choice tests included only 2 of *pomilia* inseminated by a Species A partner, while 10 *pomilia* were inseminated by *pomilia* partners. There was also a bias toward homozygotic pairings on the Species A side, yielding a

Table 1. Copulations observed in the two mate choice experiments, *Physa* Species A × *P. acuta* (above) and *Physa* Species A × *P. pomilia* (below).

		Males		
		Homogametic	Heterogametic	Totals
Females	Species A (S)	15	15	30
	P. acuta (A)	12	7	19
				49
Females	Species A (S)	16	10	26
	P. pomilia (H)	10	2	12
				38

significant overall deviation from random mating (normalized $\chi^2 = 6.63$, $p = 0.01$).

Reared together in a no-choice design, mixed pairs of Species A and *P. acuta* showed no delay in age at first reproduction, their modal age at maturation (7 wks) indeed slightly less than that observed in either matched Species A or matched *acuta* control pairs (Table 2). A reduction was apparent in parental fecundity, however, the median of 55.2 embryos/wk posted by SA outcross pairs significantly below both controls ($p = 0.027$). The 73.1% median viability of the F1 Species A / *acuta* hybrids was intermediate between the F1 viabilities observed from incross controls.

Electrophoretic analysis of a sample of offspring from three SA outcrosses confirmed the hybridity of all F1 progeny. One pair of parents was fortuitously fixed for alternative alleles at the Isdh locus, yielding a sample of twelve entirely heterozygous progeny. A second pair of SA parents were both heterozygous at the Est3 locus ($Est3^{100}/Est3^{96} \times Est3^{96}/Est3^{92}$), yielding twelve F1 progeny in four classes. The third pair of parents included one heterozygote at the Isdh locus ($Isdh^{100}/Isdh^{100} \times Isdh^{100}/Isdh^{97}$), and one heterozygote at the Est3 locus ($Est3^{100}/Est3^{92} \times Est3^{92}/Est3^{92}$), yielding at both loci twelve F1 progeny representing the heterozygous and one homozygous class, missing the other homozygous class entirely. The likelihood of missing a single homozygous class in twelve selfed progeny from a heterozygous parent would be 0.032.

None of the nine pairs of F1 progeny from the SA outcross produced viable F2 offspring. One SA pair was terminated early by mortality, while the other eight pairs all laid eggs profusely, beginning at week 7 and extending to week 19. All egg masses laid by all eight pairs of SA hybrids over the 12 week period were held for five weeks, with no hatching observed.

Reared together in a no-choice design, pairs of Species A and *P. pomilia* demonstrated significant delays in age at first reproduction behind that posted by their combined controls (Fisher's exact $p = 0.003$). Their modal age of 9 weeks at the onset of egg laying was slightly behind both the Species A control and the *pomilia* control (Table 3). The median parental fecundity of 27.3 embryos/wk posted in the SH outcross experiment was also significantly lower than both incross controls ($p = 0.002$), and the median viability of their progeny (64.8%) lower than the Species A control. Only one of the nine pairs of first generation progeny from the SH experiment yielded viable second generation offspring, at week 20. Most of the remaining first generation pairs laid eggs that failed to hatch, generally over many weeks of observation.

Electrophoretic analysis revealed that two sets of SH parents were fortuitously fixed for alternative alleles at the LAP locus. Samples of ten first generation progeny from both of these crosses yielded only one homozygous class, strongly suggesting self-fertilization by the Species A parent, and no reproduction by the *pomilia* parent. Absence of suitable genetic markers made inference regarding the third set of SH progeny analyzed equivocal.

Table 2. Statistics comparing the fitness of *Physa* Species A × *P. acuta* outcrosses to pure *Physa* Species A and pure *P. acuta* controls.

	Species A	SA outcross	*P. acuta*
First oviposition, P generation			
Mode (weeks post hatch)	8	7	8
Range	7–8	7–10	5–10
Weekly mean parental fecundity			
Median (embryos)	75.4	55.2	66.9
Range	19–105	18–81	17–104
Weekly mean F1 viability			
Median (%)	80.5	73.1	61.7
Range	66–91	49–99	45–86
F1 Fertility	67%	0%	100%
Viable F2 hatch			
Median (week)	12.5	–	9
Range	4–19	–	9–11

Table 3. Statistics comparing the fitness of *Physa* Species A × *P. pomilia* outcrosses to pure *Physa* Species A and pure *P. pomilia* controls.

	Species A	SH outcross	*P. pomilia*
First oviposition, P generation			
Mode (weeks post hatch)	8	9	7
Range	7–8	8–12	7–11
Weekly mean parental fecundity			
Median (embryos)	75.4	27.3	57.4
Range	19–105	7–41	19–68
Weekly mean F1 viability			
Median (%)	80.5	64.8	55.8
Range	66–91	10–86	33–86
F1 Fertility	67%	10%	78%
Viable F2 hatch			
Median (week)	12.5	20	5
Range	4–19	–	3–8

DISCUSSION

The experiments reported here confirm reproductive isolation between the "*Physa* Species A" of Wethington (2004) and populations representing both of the other physid species inhabiting South Carolina, *P. acuta* and *P. pomilia*. An initiative to formally describe Species A has just been published (Wethington et al., 2009). The reproductive isolation displayed by these three species is of different degrees, however, and apparently more closely related to their genetic divergence than to their reproductive anatomy.

Physa Species A and *P. acuta* cluster in the same mtDNA phylogroup (Wethington and Lydeard, 2007) but differ in their penial anatomy. The mate choice tests reported here yielded no evidence of sexual isolation between them. A significant reduction in the joint fecundity of Species A × *P. acuta* outcross pairs was indeed revealed by no-choice breeding experiments, although there was no evidence of reduced viability in the F1 hybrids such crosses produced. Species A × *acuta* hybrids were, however, entirely sterile.

Physa Species A and *P. pomilia* share identical type-bc penial anatomy, but are more distantly related genetically. The reproductive isolation that Species A and *pomilia* display under controlled conditions is of a greater degree than that observed between Species A and *acuta*. Paired in a no-choice design, Species A and *pomilia* parents displayed delayed reproduction, reduced fertility, and reduced offspring viability. All the viable first-generation progeny recovered from SH outcrosses were attributable to self-fertilization by the Species A parent, reproduction by the *pomilia* parent apparently foreclosed. It seems likely that the substantial reduction in reproductive success demonstrated in the second generation by SH offspring may be attributable to Species A inbreeding depression. Such results are quite simiar to those we obtained from crosses between the type-c *Physa acuta* and the type-b *Physa gyrina* (Dillon et al., 2004).

In addition, mate choice tests returned evidence of prezygotic reproductive isolation between Species A and *P. pomilia*. Physids seem to mate according to a modified "Bateman's Principle" (Bateman, 1949). They are generally quick to copulate as males, and display little discrimination, but when mounted in the female role they can be choosy, often displaying rejective behaviors like evasion and shell-shaking (DeWitt, 1991; 1996; Wethington & Dillon, 1996; McCarthy and Sih, 2008). Only 38 of the 60 snails tested in the SH mate choice experiments were ultimately able to mate as males, at least partly because of the high frequency of rejective behaviors they encountered in heterogametic couplings. Our observation that *pomilia* copulants were more rejective of heterogametic insemination than Species A copulants may be related to our separate observation that the Species A partner in our SH no-choice experiments retained the ability to reproduce by self-fertilization, while the *pomilia* partner apparently did not. This situation is similar to that we have previously described for the interspecific pairing of *Physa acuta* and *P. pomilia* (Dillon et al., 2007).

Although nothing is known about the genetics of reproductive isolation in pulmonate snails, a large body of research has confirmed that both prezygotic and postzygotic barriers are inherited in a complex and polygenic fashion in *Drosophila* (Wu and Palopoli, 1993; Ritchie and Phillips, 1998). In general it has been found that postzygotic isolating mechanisms evolve independently of, and tend to lag behind, prezygotic mechanisms (Coyne and Orr, 2004). Whether the latter can be reinforced by natural selection on the former is controversial. Experiments to trace the evolution of both sets of characters through the larger phylogeny of the Physidae are currently ongoing.

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