MALACOLOGIA, 2007, 49(2): 283–292

EMPIRICAL ESTIMATES OF REPRODUCTIVE ISOLATION AMONG THE FRESHWATER PULMONATE SNAILS *PHYSA ACUTA*, *P. POMILIA*, AND *P. HENDERSONI*

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ABSTRACT

Physa hendersoni collected from its type locality near Yemassee, South Carolina, and *Physa pomilia* from its type locality near Claiborne, Alabama, both display the penial morphology that has been characterized as "type-bc" by Te (1978, 1980). Mate choice tests returned no evidence of premating reproductive isolation between these two populations, and no-choice breeding experiments confirmed outcross fecundity, F1 viability and F1 fertility comparable to incross controls. Significant premating reproductive isolation was documented, however, between the *P. hendersoni* population and a population of *Physa acuta* from Charleston, South Carolina, bearing the "type-c" penial morphology. No-choice breeding experiments involving *P. acuta* and *P. hendersoni* yielded a mixture of hybrid and selfed progeny, the hybrids apparently sterile. Thus the nomen *Physa hendersoni* is a junior synonym of *P. pomilia*, whereas *P. pomilia* and *P. acuta* are distinct biological species.

Key words: Gastropoda, Pulmonata, *Physella*, speciation, mate choice, allozyme electrophoresis.

INTRODUCTION

The most influential classification system for the Physidae at present is that of George Te (1978, 1980; Burch, 1989). Te recognized approximately 40 species and subspecies of physids in North America, divided into genera and subgenera by penial morphology. Experimental breeding studies have subsequently suggested, however, that Te's estimate of specific diversity may have been too high. Among the nominal species bearing the penial morphology Te characterized as "type-c", *Physa cubensis*, *P. heterostropha*, *P. integra*, and *P. virgata* have all recently been synonymized under *P. acuta*, described from France prior to any American physid (Dillon et al., 2002, 2005a; Paraense & Pointier, 2003). Among the nominal species bearing Te's "penial complex type-b," recent research has suggested that *P. ancillaria*, *P. aurea*, *P. microstriata*, *P. parkeri*, *P. sayii* and *P. utahensis* may all be junior synonyms of *P. gyrina* (Dillon & Wethington, 2006a, b). Reproductive isolation seems to be complete, however, between physids bearing type-b and type-c penial complexes (Dillon et al., 2004).

No attention has yet been directed, however, toward reproductive relationships in physids bearing the penial complex characterized by Te (1978) as "type-bc," intermediate between the two more common morphologies discussed above. Te attributed the type-bc penis to a set of four species inhabiting the American South, including *Physa hendersoni*, originally described by Clench (1925) as a subspecies of *P. pomilia* (Conrad, 1834). Te's observations suggested to him that *P. pomilia* had a type-c penis, however, which led him to propose that *P. pomilia* be considered a subspecies of the widespread *P. heterostropha*, which has subsequently been synonymized under *P. acuta*. Te raised *P. hendersoni* to the full species level, listed its range as extending from "West Virginia, Tennessee, and Missouri south to the Carolinas, Mississippi, and Florida", and recognized several subspecies within it (Burch, 1989).

Wethington (2003) has reported, however, that specimens freshly collected from the type locality of *Physa pomilia* bear a type-bc penial complex, not type-c as suggested by Te. Molecular data further demonstrate a close genetic similarity between *P. hendersoni* and *P.*

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pomilia, as originally proposed by Clench, and do not suggest an especially close affinity between *P. pomilia* and *P. acuta* (Wethington & Lydeard, in press). The purpose of the present work is to use experimental methods to test for reproductive isolation between three populations of physids – *Physa hendersoni*, *P. pomilia*, and *P. acuta* – the first two bearing type-bc penial morphology, the third a type-c physid, with which *P. pomilia* has been confused.

METHODS

The type locality given by Draparnaud (1805) for *Physa acuta* was the "River Garonne," that given by Conrad (1834) for *P. pomilia* was "Randon's Creek, near Claiborne, Alabama," and that given for *P. pomilia hendersoni* by Clench (1925) was "Yemassee, Beaufort Co., South Carolina." Our line "H" was established from snails collected in the Combahee River at the US 21/17A bridge, 1 km E of Yemassee, Hampton County, South Carolina (32.7060°N, 80.8281°W). Line "P" was founded from snails collected in Randons Creek at the CR 23 bridge, 12 km S of Claiborne, Monroe County, Alabama (31.4387°N, 87.5445°W). Our control line of *Physa acuta* ("A") was established from snails collected in the main pond at Charles Towne Landing State Park, west of the Ashley River, within the city limits of Charleston, South Carolina (32.8062°N, 79.9862°W). Snails of this population are not reproductively isolated from near-topotypic *P. acuta* populations sampled from France (Dillon et al., 2002). The Charleston population has previously been designated "Ctl" by Dillon & Wethington (1995), "A" by Dillon et al. (2004), and "C" by Dillon et al. (2005a). The habitat has been described by Dillon & Dutra-Clark (1992).

Wild-collected adult snails were returned to the laboratory and isolated in 10 oz. (210 ml) clear polyethylene drinking cups of aerated, filtered pond water with Petri dish covers, and fed a commercial *Spirulina*-based flake fish food, finely ground. All culture took place at approximately 23°C in a 12:12 light cycle. Every two or three days, each snail was transferred to a fresh cup, with feeding and water change, leaving egg masses attached to the walls of the previous cup. The hatchlings from these egg masses, conceived in the wild but laboratory born, served as experimental animals both for our mate choice tests and as the parental generation for our no-choice tests for reproductive isolation.

We performed two separate mate-choice experiments to assess prezygotic reproductive isolation, one comparing populations A and H, and a second comparing populations H and P. Each experiment involved 30 adult snails per population. Thus, we reared to maturity 30 snails from lines P and A, and 60 snails from line H, isolated in individual cups with weekly feeding and water change. The experiments (designated AH and HP) were both composed of three trials, involving ten snails from each population per trial, similar in shell size, marked with tiny spots of contrasting nail varnish. The 20 snails were introduced simultaneously into a two-liter glass beaker containing 1,400 ml of filtered pond water, placed on a glass countertop to facilitate observation on all sides, including the bottom.

Each trial was monitored for six hours. When a copulation was observed, defined as the complete insertion of the penis of one partner into the mantle cavity of a second, both snails were removed and their lines of origin noted. Then the shell of the individual serving as male was marked with a dot of white correction fluid, and both snails returned to the beaker. Only the first mate choice of each snail copulating as a male was recorded. Subsequent copulations undertaken by an individual already marked with white fluid were allowed to proceed undisturbed. Thus, the maximum number of data recorded for each trial was 20, although most trials concluded before all 20 individuals had copulated in the male role. Observations were combined across the three trials for each of the two experiments, AH and HP, and tested for evidence of sexual isolation with a chi-square statistic, normalized by 4/N (Gilbert & Starmer, 1985).

We also performed two separate no-choice experiments for postzygotic reproductive isolation, one between lines A and H, and the second between lines H and P. Each experiment involved the offspring of ten different wild-collected snails, randomly chosen at approximately 2 mm shell length, well before maturity. These juveniles (the parental generation) were paired in cups with individuals of a second population, for example A1 x H1, A2 x H2, …, A10 x H10 and H1 x P1, H2 x P2, …, H10 x P10. Two sets of incross controls were established simultaneously with each set of outcrosses, for example A1 x A2, A2 x A3, …,

A9 x A10, A10 x A1, and H1 x H2, H2 x H3, ..., H10 x H1. Thus, both experiments were initiated with 30 pairs of unrelated parental snails: 10 outcrosses and 20 incross controls.

All parental pairs were fed each week, and their water changed. When egg masses were observed, the embryos were counted and the pair of adults advanced to a fresh cup. The viable, crawling F1 hatchlings in each cup were enumerated two weeks subsequently. Observations on any pair of snails were terminated upon the death of either partner. Experiments proceeded for ten weeks beyond the week that a minimum of three pairs of parents first laid viable embryos, determined separately for the outcross and its two corresponding incross sets.

Only a small subset of the F1 progeny were retained and reared – the offspring of three unrelated pairs of parents per set, for example A1 x H1, A2 x H2 and A3 x H3 or A1 x A2, A3 x A4, and A5 x A6. Because both the AH experiment and the HP experiment involved an outcross and two incrosses, a total of 2 x 3 x 3 = 18 F1 sibships were retained, each sibship composed of up to ten cups of graded age. Randomly selected 2 mm individuals were paired between F1 sibships within sets, for example, AH1 x AH2, AH2 x AH3, AH3 x AH1. These F1 x F1 crosses were performed three times, as the F1 generation aged. So there were nine pairs of F1 animals for each set – three early, three middle, and three late – for a total of 9 x 3 = 27 such pairs for the AH experiment and 27 pairs for the HP experiment. These were reared to adulthood, with the week at first production of viable F2 hatchlings recorded.

At least 30 additional F1 progeny from the AH and HP outcrosses were reared to adulthood and their hybrid status confirmed via allozyme electrophoresis. We have previously identified 12 allozyme-encoding loci at which *Physa* populations commonly display polymorphisms interpretable as the product of codominant alleles, segregating in Mendelian fashion (Dillon & Wethington, 1994, 1995, 2006b; Wethington & Dillon, 1991). These are aconitase (ACON), esterases (EST, 3 loci), glucose phosphate isomerase (GPI), isocitrate dehydrogenase (ISDH, 2 loci), leucine aminopeptidase (LAP), mannose phosphate isomerase (MPI), 6-phosphogluconate dehydrogenase (6PGD), and phosphoglucomutase (PGM, 2 loci). Detailed electrophoretic methods are available in Dillon (1992).

Evidence for reproductive isolation was assessed by comparing the fitness of the outcross pairs to the incross controls using five statistics: the age at first parental oviposition, the average weekly production of embryos by parents, the proportion of the embryos hatching into viable F1 juveniles, the proportion of the F1 pairs producing F2 embryos, and the age at which the first viable F2 progeny hatched. Significant depression demonstrated by the outcross below both of the controls in any of these broad measures of fitness would constitute evidence of postzygotic reproductive isolation.

Any difference in the central tendency of age at first oviposition between the ten outcross pairs and either set of ten incross controls was tested by calculating a combined (20-pair) median and comparing counts above and below that median using Fisher's exact tests.

The embryos laid by each pair of snails were summed, then divided by the total weeks of record (ignoring prematurity zeros and postmortem zeros) to derive an average weekly fecundity for each pair of parents. Then the central tendency in average weekly fecundity across the ten outcross pairs was compared to the two corresponding incross controls using Kruskal-Wallis nonparametric analysis of variance, with two degrees of freedom. *Post hoc* analysis, if appropriate, was performed with a set of Wilcoxon rank sum tests. The statistical analysis of F1 viability was similar to that for parental fecundity. The total viable hatchlings produced by each pair of parents was divided by their total embryos laid to obtain an overall percent hatching success for each pair. Then the central tendencies in hatching success across the ten outcross pairs and the two sets of ten incross controls were tested for significant differences using Kruskal-Wallis nonparametric ANOVA, with Wilcoxon *post hoc* tests if appropriate.

Differences in the central tendency of the date at which the first viable F2 progeny hatched were tested using statistical methods analogous to those described above for age at first reproduction in the parental generation. In this case, the week at which each of the (typically 9) F1 x F1 pairs yielded their first viable, crawling juveniles was recorded, a combined (18 pair) median calculated across both of the sets to be compared, and any difference in the number of observations above and below that median assessed using Fisher's exact tests.

RESULTS

Our AH mate choice tests revealed significant premating reproductive isolation between *Physa hendersoni* collected from its type locality and control *P. acuta* collected from Charleston (Table 1, upper). The effect appeared mutual, snails of line A over three times more likely to mate as males with other line A snails, and H snails more than twice as likely to mate as males with other H animals. Only 41 copulations were observed in the 6 hours allotted (of 60 possible), 30 of which were homogametic (normalized $\chi^2 = 6.86$, $p = 0.009$). The complex interactions among non-reciprocally mating simultaneous hermaphrodites have been described by Wethington & Dillon (1996).

Mating was much more frequent in the HP mate choice tests, with 57 (of 60) snails ultimately mating as male (Table 1, lower). No evidence was detected of reproductive isolation between *P. hendersoni* and *P. pomilia*, however, lines H and P apparently mating randomly (normalized $\chi^2 = 0.74$, $p = 0.39$).

Reared together in a no-choice design, *Physa acuta* / *P. hendersoni* pairs demonstrated slight, nonsignificant delays in age at first reproduction ($p = 0.179$, Table 2). The H controls tended to initiate oviposition at an earlier age and die at an earlier age under our experimental conditions, only four control pairs and two outcross pairs surviving the entire 10-week experiment (Fig. 1). Mean weekly parental fecundity was lower in the AH outcross than in the controls, although again this was not significant ($p = 0.08$), nor was any significant difference detected in F1 viability ($p = 0.76$). Fertility was, however, strikingly reduced in the F1 generation reared from the AH outcross, with only four (of nine) F1 pairs ultimately yielding viable F2 progeny, in widely scattered weeks. The five F1 pairs failing to reproduce included two pairs that laid infertile eggs for 8–9 weeks.

The reproductive delays and reduced fecundities demonstrated in the AH outcross, together with an (apparently natural) accelerated mortality in *Physa hendersoni* adults, combined to yield only 36 F1 progeny (from two sibships) for hybrid testing via allozyme electrophoresis. A fixed difference between the parents of both sibships at the LAP locus allowed unambiguous classification of the 36 individuals as eight offspring from self-fertilization by the *P. acuta* parent, ten offspring from self-fertilization by the *P. hendersoni* parent, and 18 hybrids.

None of our tests comparing *Physa hendersoni* and *P. pomilia* returned evidence of postzygotic reproductive isolation, although we did detect significant life history differences between the control lines (Table 3). *Physa pomilia* control pairs initiated oviposition significantly earlier than either our *P. hendersoni* controls or our HP outcrosses (Fisher's exact $p = 0.001$ in both cases) with good survivorship over ten weeks of reproduction in all three sets (Fig. 2). Similarly, *P. pomilia* parents demonstrated greater mean weekly fecundity (Kruskal-Wallis $\chi^2 = 8.07$, $p = 0.018$), their 69.4 embryos/week significantly higher than both the 49.2 posted by the *P. hendersoni* controls (Wilcoxon $p = 0.043$) and the 52.6 posted by the HP outcross pairs (Wilcoxon $p = 0.003$). No significant differences were detected between the HP outcross and the *P. hendersoni* control in either age at first reproduction or in parental fecundity. Nor were differences detected among all three sets in F1 viability, F1 fertility, or viable F2 hatch.

TABLE 1. Copulations observed in the two mate choice experiments, *P. acuta* x *P. hendersoni* (above) and *P. hendersoni* x *P. pomilia* (below).

		Males			
		Homogametic	Heterogametic	Totals	
Females	*P. acuta* (A)	17	5	22	
	P. hendersoni (H)	13	6	19	
					41
Females	*P. hendersoni* (H)	12	10	22	
	P. pomilia (P)	20	15	35	
					57

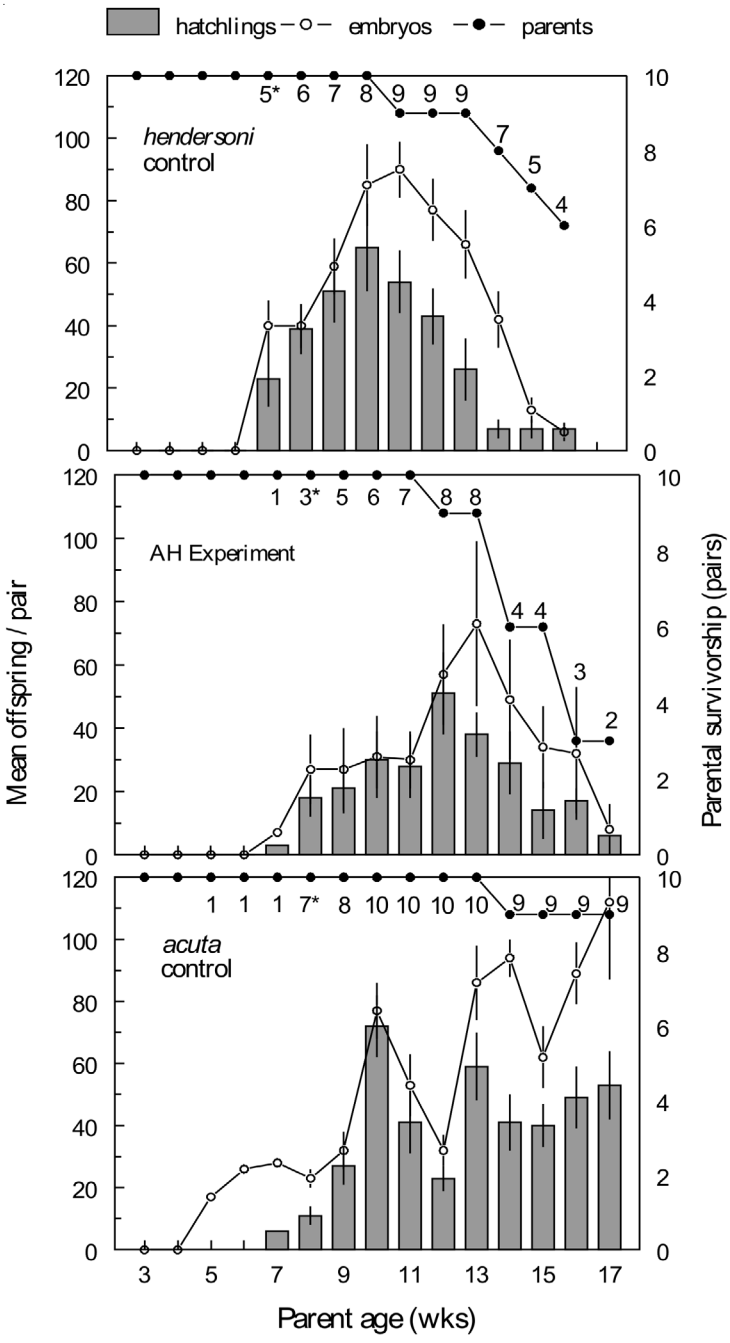

FIG. 1. Production of embryos and viable hatchlings as a function of parental age (weeks post-hatch) in the *P. acuta* x *P. hendersoni* (AH) outcross experiment, pure *P. hendersoni* controls, and pure *P. acuta* controls. The bars are standard errors of the mean. The number of reproducing pairs is given with parental survivorship (right axis). Asterisks* denote week 1 for analysis of variance.

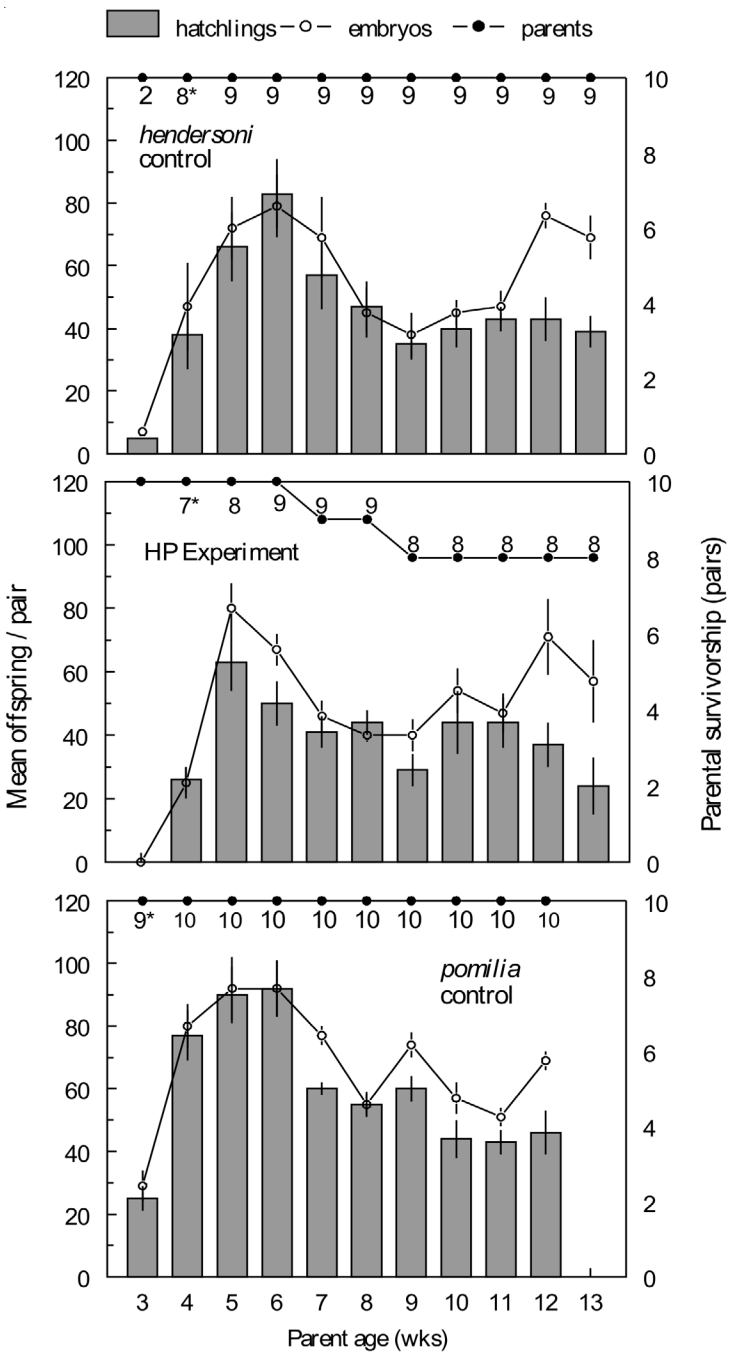

FIG. 2. Production of embryos and viable hatchlings as a function of parental age (weeks post-hatch) in the *P. hendersoni* x *P. pomilia* (HP) outcross experiment, pure *P. hendersoni* controls, and pure *P. pomilia* controls. The bars are standard errors of the mean. The number of reproducing pairs is given with parental survivorship (right axis). Asterisks* denote week 1 for analysis of variance.

TABLE 2. Statistics comparing the fitness of *P. acuta* x *P. hendersoni* outcrosses to pure *P. acuta* and pure *P. hendersoni* controls.

	P. acuta	AH outcross	*P. hendersoni*	p
First oviposition, P generation				0.179
Mode (week)	8	9	7	
Range	5–10	7–16	7–11	
Weekly mean parental fecundity				0.08
Median (embryos)	66.9	24.8	57.4	
Range	17–104	3–96	19–68	
Weekly mean F1 viability				0.756
Median (%)	61.7	67.1	55.8	
Range	45–86	0–91	33–86	
F1 Fertility (%)	100%	44%	78%	
Viable F2 hatch				-
Mode (week)	9	-	5	
Range	9–11	-	3–8	

Electrophoretic analysis of a sample of offspring from three HP outcrosses confirmed the hybrid status of all F1 progeny. Two pairs of parents were (fortuitously) fixed for alternative alleles at the LAP locus, yielding a sample of ten entirely heterozygous progeny in both cases. The progeny of the third pair included six FF homozygotes and six FS heterozygotes, which significantly differs from the 1:2:1 selfed expectation. (The likelihood of sampling no SS homozygotes in 12 trials from such a sibship is 0.032).

DISCUSSION

The data presented here do not support the elevation of *Physa hendersoni* to full specific level (Te, 1978; Burch, 1989). Rather, they confirm the initial proposal of Clench (1925) that the populations described by him as *P. hendersoni* are not specifically distinct from *P. pomilia*. Table 1 demonstrates random mating between snails of our lines H and P. Control populations of *P. pomilia* initially reproduced at an earlier age and showed sig-

TABLE 3. Statistics comparing the fitness of *P. hendersoni* x *P. pomilia* outcrosses to pure *P. hendersoni* and pure *P. pomilia* controls.

	P. hendersoni	HP outcross	*P. pomilia*	p
First oviposition, P generation				0.001
Mode (week)	4	4	3	
Range	3–5	4–6	3–4	
Weekly mean parental fecundity				0.018
Median (embryos)	49.2	52.6	69.4	
Range	0–91	41–70	58–80	
Weekly mean F1 viability				0.128
Median (%)	82.1	78.2	90.2	
Range	0–95	61–97	74–98	
F1 Fertility (%)	100%	100%	100%	
Viable F2 hatch				0.143
Mode (week)	6	6	6	
Range	6–7	5–9	6–7	

nificantly greater fecundity than control *P. hendersoni* under our experimental conditions, but the performance of the HP outcross was not different from that of the *P. hendersoni* control (Table 3). No difference was apparent between the outcross and either of the controls at the F1 or F2 generations.

The distinctions that Clench noted between his new subspecies *Physa pomilia hendersoni* and *P. pomilia* (s.s.) were entirely with regard to minor aspects of shell morphology, the former displaying, "a shorter aperture as compared to the total length of the shell; the aperture more rounded; the spire more produced and acute, and the sutures more deeply impressed." We were not able to confirm these differences, snails from lines H and P being completely indistinguishable to our eyes in culture. It seems likely to us that that the shell differences initially prompting Clench's description of subspecies *hendersoni* may have been ecophenotypic in origin.

Nor do our data support the hypothesis of Te and Burch that *Physa pomilia* should be considered subspecific to *P. heterostropha*, now understood to be a junior synonym of the cosmopolitan *P. acuta*. Rather, our results demonstrate that *P. pomilia* and *P. acuta* are distinct biological species, displaying both prezygotic and postzygotic reproductive isolation. Half of the F1 offspring recovered from no-choice pairings of *P. acuta* and *P. pomilia* were the result of self-fertilization, whereas the rate of self-fertilization is typically found to be negligible in matched controls (Wethington & Dillon, 1993, 1997; Dillon et al., 2005b). Our data further suggest that the other half of the F1 offspring, those that were hybrids, may have been sterile.

At the initiation of our F1 x F1 tests we had no knowledge regarding the hybrid status of the individuals chosen, and hence the nine pairs of F1 snails we selected from the AH lines to assess the production of a viable F2 generation would be expected to include roughly equal proportions of selfed and hybrid animals. Three F1 pairs died relatively early in the experiment, without issue. Two pairs yielded viable F2 offspring early in the experiment, both of which were subsequently shown by allozyme electrophoresis to be composed of one pure-A animal and one pure-H animal. Two pairs yielded viable F2 offspring late in the experiment, both of which proved to be one pure-A animal and one hybrid. And two pairs were terminated late in the experiment by the death of a parent, after weeks of laying infertile eggs. The surviving F1 animal was shown to be a hybrid in both cases. Thus, all viable F2 offspring ultimately recovered from the AH experiment are attributable to reproduction by purebred F1 animals, and our results are consistent with a hypothesis of *P. acuta* x *P. pomilia* hybrid sterility.

The significant tendency of animals from our H and A lines to select mates from their own populations (Table 1, upper) is the second demonstration of prezygotic reproductive isolation in basommatophoran pulmonates of which we are aware. Similar phenomena have previously been documented in stylommatophoran pulmonates (Baur & Baur, 1992; Fearnley, 1996) as well as in the intertidal caenogastropod snail *Littorina* (Pickles & Grahame, 1999; Rolan-Alvarez et al., 1999; Cruz et al., 2004). But the only prior report of prezygotic reproductive isolation in freshwater pulmonates, to our knowledge, is that of Rupp & Woolhouse (1999) working with *Biomphalaria glabrata* and *B. pfeifferi*.

Clench gave the distribution of "typical" *Physa pomilia* as "extending as far north as Kentucky and west to Mississippi." He suggested that his new subspecies *P. pomilia hendersoni* "seems to be confined to the coastal area of the Southeast." Our personal observations, although more limited, tend to agree. *Physa pomilia* seems moderately common in Atlantic drainages from Virginia to Georgia, especially in coastal rivers of low current. Although it is clear that the species extends as far south as Alabama and as far north as Connecticut (Wethington & Lydeard, in press), we do not have good field records beyond the southern Atlantic drainages. The situation is complicated by confusion with *P. acuta*, which is common and widespread throughout North America, and which can be difficult to distinguish from *P. pomilia* without dissection. A third species of physid also inhabits swampy coastal environments of the American Southeast and may also be confused with *P. pomilia*. Work to characterize this third species is currently ongoing.

Our data on reproductive isolation agree well with the results of recent studies reporting mitochondrial sequence divergence in large samples of physids surveyed from throughout North America (Wethington & Guralnick, 2004; Wethington & Lydeard, in press). Molecular phylogenetic analysis has confirmed that *Physa hendersoni*, *P. pomilia*, and other type-bc physids are closely related, and that type-bc physids are genetically distinct from both

the type-b and type-c groups. Additional studies to characterize reproductive relationships in earlier branches of the phylogeny could yield valuable insight into the mechanics of speciation, not just in the Physidae but throughout the invertebrate fauna of fresh waters.

ACKNOWLEDGEMENTS

We thank Deborah L. Kirkland for field assistance and Tom Smith for help with snail culturing chores. This research was supported by a grant from the National Science Foundation, DEB-0128964.

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Revised ms. accepted 29 September 2006