# ALLOZYME, 16S, AND CO1 SEQUENCE DIVERGENCE AMONG POPULATIONS OF THE COSMOPOLITAN FRESHWATER SNAIL, *PHYSA ACUTA*

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Running Title: Population divergence in an invasive snail

Updates 2024:

This paper, originally written in 2004 but not ultimately published until 2022, reports the discovery of a mitotype associated with cytoplasmic male sterility (CMS) in *Physa* twenty years before that phenomenon was understood. See the FWGNA Blog for additional details:

- Cytoplasmic Male Sterility in *Physa* [9June22]
- Cytoplasmic Male Sterility in the Snake River *Physa* [7Aug24]

The sequences discovered in Charleston associated with CMS were uploaded to GenBank in September of 2024 as follows:

| **Snail** | **16S** | **CO1** |
|---|---|---|
| **C10** | PQ275512 | PQ272431 |
| **C13** | PQ275513 | PQ272432 |
| **C15** | PQ275514 | PQ272433 |
| **C18** | PQ275515 | PQ272434 |

## Abstract

We document genetic divergence among six populations of an invasive freshwater pulmonate snail, the European *Physa acuta* and two of its North American synonyms, *P. heterostropha* and *P. integra.* Our analysis uncovered 73 unique CO1 haplotypes in 98 individuals and 81 unique 16S haplotypes in 124 individuals. A molecular phylogeny based on the combined CO1+16S sequence returned a monophyletic *acuta* group, phylogenetically distinct from outgroups *Physa gyrina* and *P. hendersoni*, confirming that the six populations constitute a single phylogenetic species as well as a biological one. Four individuals bearing a strikingly divergent haplotype were discovered in a population from South Carolina, with a genetic distance of approximately 30% from all other *P. acuta*. Haplotype diversity was high even with these outliers removed, however, ranging up to 10.3% within an Irish population and 13.3% in a population from Michigan ($F_{SC}$ = 0.584, $F_{ST}$ = 0.557). Genotype frequencies at seven polymorphic allozyme-encoding loci were generally in conformance with Hardy-Weinberg expectation, occasionally showing private alleles at high frequency. Mean heterozygosity ranged from 10.7% in Michigan to 25.8% in Philadelphia, and interspecific Nei genetic distances ranged from 0.022 to 0.843 ($F_{IS}$ = 0.104, $F_{ST}$ = 0.472). No analysis revealed any reduction in the genetic diversity of European *P. acuta* populations below that observed in America, nor was any component of the variance attributable to continental subdivision ( $F_{CT}$ = -0.065 sequence, $F_{CT}$ = -0.027 allozymes), suggesting that if the species did in fact spread from America during historical times, no genetic bottleneck was involved.

## Introduction

Freshwater pulmonate snails of the family Physidae are among the most abundant and widespread elements of the benthic macroinvertebrate fauna of North America (Brown 1991; Dillon 2000). They have figured prominently in many studies of general importance to our understanding of aquatic ecosytems (Clampitt 1970; Brown 1982; Kesler *et al*. 1986; Sheldon 1987; Osenberg 1989; Dillon & Davis 1991; Hershey 1992; Martin *et al*. 1992; McCollum *et al*. 1998), predator-prey interactions (Thorp & Bergey 1981; Stein *et al*. 1984; Crowl & Covich 1990; Hanson *et al*. 1990; Alexander & Covich 1991; McCarthy & Fisher 2000; DeWitt *et al*. 1999; DeWitt *et al*. 2000), population genetics (Buth & Suloway 1983; Liu 1993; Liu & Mitton 1993; Dillon & Wethington 1995; Bousset *et al.* 2004) and life history evolution (Brown 1979; Rollo & Hawryluk 1988). By virtue of their reproductive diversity and ease of culture, physids have become a model organism for the study of sex allocation (Wethington & Dillon 1991; 1993, 1996, 1997; DeWitt 1991, 1995; and Jarne *et al*. 2000).

Despite the prominence of physid snails in the literature of ecological and evolutionary biology, the taxonomy of the Physidae remains in a confused state. The original descriptions of most species were based solely on minor differences in shell shape, size, or color. Physid shell morphology can, however, be affected by predator cues (Crowl 1990; Crowl & Covich 1990; McCarthy & Fisher 2000; DeWitt *et al*. 1999; DeWitt *et al*. 2000), parasitism (Wilke & Falniowski 2001), and various other environmental factors (Burnside 1998; Britton in press). Te's (1975, 1978, 1980) influential reassessment of the North American Physidae, based partially on soft anatomy, recognized 41 nominal species and 40 subspecies or geographic variants (Burch 1982; Burch 1988; Burch & Tottenham 1980; Turgeon *et al*. 1998).

Most of the species that have played important rolls as model organisms for ecological or

evolutionary research were placed by Te in the subgenus *Costatella*, including *Physa acuta* (Draparnaud 1805) of western Europe, *P. heterostropha* (Say 1817) of eastern North America, and *P. integra* (Haldeman 1842) of the American midwest. These nominal species show great phenotypic variability throughout their fluid ranges, the invasive *P. acuta* being freshly reported from northern Europe, the Middle East, Africa, North and Central America, Hawaii, Australia, New Zealand, Fiji and Japan in the last 100 years (Abbott 1950; Beetle 1973; Te 1978; Brandt 1980; Kristensen & Ogunowo 1992; Cowie 2001). Several investigators have suggested that *P. acuta* might not be a European native, but may instead have been introduced from North America where physid diversity is at its greatest (Te 1978; Brown 1980).

Dillon *et al*. (2002) intercrossed lines of *Physa* from the type or near-type localities of *P. acuta*, *P. heterostropha*, and *P. integra* both with each other and with nominally conspecific populations isolated at distances of 500 km. The reproductive performance of the six outcross populations was generally intermediate between the incross controls in age at first parental reproduction, parental fecundity, $F_1$ viability, and $F_1$ fertility, and in no case significantly worse than the more poorly-performing control. The absence of any evidence of postzygotic reproductive isolation, together with previous findings of negligible prezygotic reproductive isolation (Wethington *et al*. 2000), prompted Dillon and his colleagues to synonymize *P. heterostropha* and *P. integra* under the older nomen *P. acuta*. The authors suggested that *P. acuta*, now more broadly understood to include American populations, might be the world's most cosmopolitan freshwater gastropod.

Since Rosen (1979) asserted that reproductive compatibility is a plesiomorphic attribute, the "phylogenetic species concept" and related concepts have been offered as alternatives to the biological species concept employed by Dillon and his colleagues (de Queiroz & Donoghue

1988, 1990; Mishler & Donoghue 1982; Donoghue 1985; Mishler & Brandon 1987). Harrison (1998) has suggested that reproductive isolation may best be understood as a mechanism causing speciation, rather than as a character diagnostic of the species. But since advocates of the phylogenetic species concept emphasize the individual organism as the unit of evolution, while advocates of the biological species concept emphasize populations, a compromise does not seem imminent.

The purposes of this research are threefold. First, we assess the levels of mitochondrial and allozyme frequency variation within and among the six populations of *P. acuta* involved in the Dillon *et al*. (2002) breeding experiments, examining genetic diversity in an invasive organism which, despite obvious biological limitations, has spread over six continents. Second, we prospect for evidence that might confirm a North American origin for *P. acuta*. Third, we examine the extent to which the phylogenetic species concept corresponds to the biological species concept in this unique situation.

## Materials and methods

*Study Populations*

Wild-collected specimens were analyzed from the six populations used to found the laboratory lines of Dillon *et al*. (2002). *Physa acuta* population F was sampled near the type locality for the species, the Rieutort Wadi in Saint-Martin de Londres, 25 km north of Montpellier, France. A second population of European *P. acuta* (population I) was sampled from the Glastry Clay Pits, County Down, Northern Ireland. Population P was collected from a near-type locality for *Physa heterostropha*, the Schuylkill River at Fairmount Park in Philadelphia, Pennsylvania. A second population of nominal *P. heterostropha* (population C) was sampled from the pond at Charles Towne Landing State Park, in Charleston, South Carolina. Population N was sampled from the type locality of *Physa integra*, a small pond and connecting stream at the historical site of New Harmony, Posey County, Indiana. A second sample of nominal *P. integra* (population D) was collected from Douglas Lake at the University of Michigan Biological Station, Cheboygan County in northern Michigan. We also sampled one population of *P. gyrina* (G) from Little Lake at the Escanaba State Forest Campground, Marquette County, Michigan and one population of *P. hendersoni* (Y) from its type locality on the Salkehatchie River at Yemassee, South Carolina to serve as outgroups. Initial identifications based on shell morphology were confirmed by dissection and examination of the penial complex, which is "type c" for *Physa acuta*, (Paraense & Pointier 2003), "type b" for *P. gyrina*, and "type bc" for *P. hendersoni* (Te 1975, 1978, 1980).

*Mitochondrial DNA sequence*

Specimens for DNA sequencing were either frozen whole or stored in 95% ethanol before DNA

isolation. Sample sizes for DNA sequences ranged from 12 (population P) to 24 (population C). Genomic DNA was isolated from head tissues or whole animal using standard phenol/chloroform extraction. Mitochondrial DNA sequences were obtained for a 650 base pair segment of the mitochondrial cytochrome oxidase c subunit 1 (CO1) (Folmer *et al*. 1994) and a 550 base pair segment of the mitochondrial 16S rRNA gene (Palumbi *et al*. 1991). The target genes were amplified via PCR using 50-500 ng of template genomic DNA in 25 µl volumes (10 mM Tris, 50 mM KCl, 2.5 mM $MgCl_2$, 1 µM each primer, 0.1 mM each dNTP, 1.5 units Taq DNA polymerase; Fisher Scientific). The amplification regime began with a denaturation at $92^0$C for two minutes followed by 35 cycles of the following: denaturation at $92^0$C for 40 seconds, annealing at $52^0$C for 60 seconds (16S)/ $50^0$C for 60 seconds (CO1), and extension at $68^0$C for 90 seconds. Reaction products were purified using Qiagen DyeEx spin columns and concentrated using Millipore Ultrafree MC filters. Cycle sequencing was performed on an ABI 3100 genetic analyzer using the ABI BigDye kit and the manufacturer's instructions.

Sequence data were initially aligned using Clustal W (Thompson *et al*. 1994). Comparison of 16S rRNA sequences to molluscan secondary structure models of conserved stems and loops (Lydeard *et al*. 2000) identified occasional large loop-region indels, which were excised in BioEdit (Hall 1999). Each 16S sequence was then concatenated to its corresponding CO1 sequence for a single phylogenetic analysis.

An optimal model for sequence evolution was determined using Modeltest 3.06 (Posada & Crandall 1998; copyrighted by Posada at Brigham Young University 1998-2000) and run on PAUP (Swofford 2001). The combined analysis of the six *P. acuta* populations and two outgroups resulted in 82 taxa with 1065 characters (after duplicate sequences and portions of the 16S loop region were removed). The TVM+I+G model was selected with base frequencies as

follows: A = 0.30390, C = 0.14670, G = 0.19570, T = 0.35370. The substitution model for the rate matrix was as follows: R(a) [A-C] = 1.491100, R(b) [A-G] = 2.479100, R(c) [A-T] = 1.227500, R(d) [C-G] = 1.259500, R(e) [C-T] = 2.479100,R(f) [G-T] = 1.0000. The proportion of invariable sites (I) was 0.3596 and the variable sites (G), gamma distribution shape parameter, was 0.8212. A 10,000 bootstrap replicate neighbor joining analysis was performed using the GTR+G+I model maximum likelihood as a distance measure (selected by Modeltest 3.06) using the BioNJ method.

We used Arlequin ver. 2.000 (Schneider *et al*. 2000, copyrighted by Excoffier 1995-2000) to discover whether our sequence data might contain genetic structure corresponding to continent of collection. Three initial analyses were performed within the six *P. acuta* populations - one of the CO1 data set alone, a second based on the 16S data set alone (with loop sequences unedited) and a third based on the concatenated CO1 + 16S (edited) data set as analyzed phylogenetically above. Variance in the concatenated data set was partitioned into a component among continents, a component between populations within continents, and a component within populations using analysis of molecular variance.

*Allozyme Electrophoresis*

Sample sizes for the analysis of gene frequencies at allozyme-encoding loci generally ranged from 30 to 40 individuals, except population C (from 53 to 103 individuals). Specimens were frozen in 100 – 200 microliters of tissue buffer (Tris HCL 7.4, 7% sucrose) with a xylene cyanol marker. For electrophoresis, whole-animal homogenates were centrifuged and the supernatant resolved in 14% starch gels (a 1:1 mixture of Sigma starch and Electrostarch). Four buffer systems were employed: AP6, TC6.0, TEB8 (Dillon 1992), and TC6.8 (Mulvey & Vrijenhoek

1981). Gels were sliced and stained to demonstrate 6 enzymes using standard techniques (Shaw & Prasad 1970). Glucose phosphate isomerase (GPI) was resolved on the AP6 and TC6.8 buffers, 6-phosphogluconate dehydrogenase (6Pgd) on the AP6 and TEB8 buffers, isocitrate dehydrogenase (Isdh) on AP6 and TC6.8, and leucine aminopeptidase (Lap) on AP6 and TEB8. We scored only the faster of the two loci encoding phosphoglucomutase (Pgm2) resolved on both TC6 and TC6.8. We scored two (of several) loci encoding esterases (Est3 and Est6) as resolved on TEB8 buffer (Dillon & Wethington 1994). A detailed description of our general methods for allozyme electrophoresis, including equipment employed and recipes for all buffers and stains is available in Dillon (1992).

Mendelian inheritance of Esterase, Lap, Gpi, Pgm, and 6Pgd allozyme phenotype has been previously confirmed in several pulmonate taxa (Dillon & Wethington 1994; Mulvey & Vrijenhoek 1984; Mulvey *et al*. 1988). The allele encoding the most common allozyme band in population C was designated "100," and other alleles were named by the mobilities of their products (in millimeters) relative to this standard. Then gene frequencies, mean (direct-count) heterozygosities, genic diversity analyses, and genetic distances were calculated using Biosys-1 (Release 1.7, Swofford & Selander 1981). Conformance to Hardy-Weinberg expectation was tested at all polymorphic loci by chi-square, with Yates correction in 2x2 cases, pooling within homozygous and heterozygous genotypic classes as necessary. Unbiased genetic identities and distances were calculated among all 8 populations using the method of Nei (1978), and hierarchical F-statistics estimated across the six *P. acuta* populations using the methods of Nei (1977) and Wright (1978).

## Results

*Mitochondrial DNA*

CO1 sequences from 98 individuals and 16SrDNA sequences from 124 individuals from the six populations of *P. acuta* contained 73 and 81 unique mtDNA haplotypes, respectively. All three *P. gyrina* (population G) shared the same haplotype, as did two of the three *P. hendersoni* (Y). A total of 104 CO1 sequences and 130 16s sequences have been entered into GenBank (accession numbers XXXX – XXXX). Figure 1 shows the subset of 96 individuals for which both genes were successfully sequenced clustered in a neighbor-joining tree based on the TVM+I+G likelihood distances chosen by Modeltest 3.06 (Posada & Crandall 1998). The topology confirms that *P. acuta* is monophyletic relative to outgroups *P. hendersoni* and *P. gyrina*. None of the six populations appeared to be monophyletic, however, nor did the three formerly recognized nominal species *P. heterostropha* (P and C), *P. integra* (N and D) or *P. acuta* (*sensu strictu* F and I) constitute monophyletic groups, nor did any recognizable cluster correspond to continent of collection. Rather, the most striking feature of Figure 1 is the appearance of four haplotypes from the Charleston population (C10, C15, C13, and C18) clustered well outside the main body of *P. acuta* sequences.

The four individual snails bearing these haplotypes were not morphologically distinguishable from typical *P. acuta* in any respect. Yet their haplotypes differed from those of the other 20 snails in the Charleston population by an average of 31.9% for the 16S gene, 32.6% for the CO1 gene, and 30.4% for the two sequences concatenated and edited. A BLAST search of the Genbank returned greatest similarity to 16S and CO1 sequences from other pulmonate snails already in the database, suggesting that contamination was not a factor. But because

cryptic speciation cannot be ruled out at present, we elected to delete these four outliers from the gene diversity analysis.

For the 94 CO1 sequences and 120 16SrDNA sequences remaining, Table 1 summarizes the sample size, number of haplotypes, and range of uncorrected genetic distances within each of the six *P. acuta* populations as well as the mean number of pairwise differences and nucleotide diversity for the three data sets. Totals are also given for each value mentioned above in Table 1. The populations with the greatest within population genetic variation appear to be D and I for all three data sets. Table 2 shows the results of the analysis of molecular variance based on the concatenated 16s + CO1 data set. The fixation indices were $F_{SC}$ = 0.584 within populations, $F_{ST}$ = 0.557 among populations (within continents) and $F_{CT}$ = -0.065 between continents.

*Allozyme Electrophoresis*

Gene frequencies at seven enzyme loci for our eight study populations are given in Table 3. Sample sizes were at least 30 across all 7 x 8 = 56 observations, except for the New Harmony population (N = 24 for Lap, N =28 for Isdh). Mean direct-count heterozygosities were not strikingly different among populations, ranging from 0.107 $\pm$ 0.071 in population D to 0.212 $\pm$ 0.097 in population I. By the 0.95 criterion, 26 observations may be considered to reflect polymorphism. Genotype frequencies were significantly different from Hardy-Weinberg expectation in three observations, all at the leucine aminopeptidase locus: population C (chi square = 6.03, p = 0.014), population N (chi square = 5.41, p = 0.020), and population Y (chi square = 7.12, p = 0.008). The heterozygosity observed is significantly below Hardy-Weinberg expectation in all three cases, but it is not clear whether this result reflects low levels of self-

fertilization previously documented in *Physa* populations (Wethington & Dillon 1997; Dillon *et al*. in press) or type I statistical error. Across the six populations of the *P. acuta* and seven loci, average coefficients of inbreeding were $F_{IS} = 0.104$, $F_{IT} = 0.0527$, and $F_{ST} = 0.472$. Hierarchical reanalysis, dividing the populations into four American and two European, returned an average $F_{SC}$ (populations within continents) = 0.483 and $F_{ST} = 0.469$, with negligible variance attributable to continents within the total ($F_{CT} = -0.027$).

The levels of interpopulation divergence generally appear high; *Physa gyrina* population G sharing no alleles at any locus with most populations of *P. acuta*. The Yemassee (Y) population of *P. hendersoni* appeared intermediate in some sense between the other two species, more genetically similar to *P. acuta* but sharing some alleles with *P. gyrina*. Within *P. acuta*, the northern Michigan population (D) was distinguished by high frequencies of private alleles at several loci. The matrix of Nei's statistics based on these data is given in Table 4.

## Discussion

The levels of genetic diversity within our six populations of *Physa acuta* ($F_{SC}$ = 0.584 mtDNA, $F_{IT}$ = 0.104 allozymes) as well as the genetic divergence among them ($F_{ST}$ = 0.557 mtDNA, $F_{ST}$ = 0.472 allozymes) are high by comparison to values typically reported for other organisms in the literature. But in view of the large geographic distances separating these populations and the poor overland dispersal capabilities reasonably expected from freshwater gastropods, one might be surprised that levels of genetic divergence were not greater.

Population D from northern Michigan was the most distinctive of the *P. acuta* populations on the basis of gene frequencies at allozyme-encoding loci, fixed for the unique allele $Est6^{92}$ and carrying two other unique alleles in high frequency, $Est3^{105}$ and $Isdh^{104}$ (Table 3). We detected only one fixed difference among the other five populations combined (pair N and F at Est6) and only one unique allele ($Isdh^{97}$ in population P). Genetic distances among populations N, P, C, F, and I ranged from 0.022 to 0.284 (Table 4). By comparison, the ten populations of *P. acuta* surveyed from the area around Charleston, SC, also displayed one fixed difference and two private alleles (Dillon & Wethington 1995). Nei's genetic distances among the ten Charleston populations, isolated by brackish water creeks but in no case separated by greater than 50 km, generally ranged from 0.10 to 0.25 and up to 0.33. The allozyme divergence of population D from the other five *P. acuta* populations was somewhat greater, with genetic distances ranging from 0.626 to 0.843.

The number of unique mitochondrial haplotypes we discovered in each *P. acuta* population generally approached the number of individuals sampled (Table 1). For 16S + CO1 concatenated and edited, matching sequences were generally found only within populations (2 of 12 in P, 2 of 18 in C, and 2 of 14 in I). The only exception was a single sequence shared

between the P and C populations (C8 = C17 = P6). Setting aside the population C outliers, the mean number of pairwise nucleotide differences ranged from 7.18 ± 3.58 in Population F to 35.41 ± 16.35 in Population D. Percent difference in concatenated 16S + CO1 sequence ranged up to 10.3% within population I and up to 13.3% in population D.

Similarly high levels of mitochondrial sequence diversity have recently been reported in many populations of pulmonate land snails (Thomaz *et al* 1996; Ross 1999; Davison 2000; Hayashi & Chiba 2000; Thacker & Hadfield 2000; Goodacre & Wade 2001; Teshima *et al* 2003). Thomaz and his colleagues suggested four non-exclusive factors that might contribute to this phenomenon: (1) extremely rapid mitochondrial evolution, (2) ancient isolating factors contributing to extreme mitochondrial divergence before reestablishment of panmixia, (3) natural selection favoring the polymorphism, and (4) ecological factors lending to population structuring that favors the retention of ancient haplotypes. Although genetic variation in pulmonate snails has traditionally been attributed at least partly to selection (Jones *et al* 1977; Clarke *et al* 1978), Thomaz and subsequent workers have generally favored population subdivision as the most important factor.

Despite high levels of sequence divergence, our mtDNA phylogeny supported the monophyly of *Physa acuta* (Figure 1). Thus our six populations constitute a single phylogenetic species as well as a biological one. Three (of 14) individuals from population I appeared to form a distinct cluster, as did 13 (of 15) individuals from population D. The most distinctive cluster of *P. acuta* haplotypes was, however, born by four of the 18 snails sequenced from Charleston population C. The likelihood that the pond from which these 18 snails were collected hosts an admixture of two cryptic species seems low, but not entirely negligible. Our 15 years of laboratory breeding experiments, involving scores of lines founded separately from population

C, have revealed no evidence of reproductive isolation (Wethington & Dillon 1993, 1996, 1997). Our surveys of allozyme genotype frequencies, both of 70 individuals at ten loci (Dillon & Wethington 1995) and of 50 -100 individuals at the seven loci reported here, generally show conformance to Hardy-Weinberg expectation within population C. The exception at the LAP locus seems attributable to background levels of self-fertilization (Wethington & Dillon 1997; Dillon *et al*. in press). Nevertheless, an effort to isolate and intercross lines of *Physa* from population C that have the two divergent mitochondrial haplotypes is currently underway in our laboratory.

Although the hypothesis remains quite plausible on the basis of other evidence, neither our allozyme data nor our mitochondrial sequence data support previous suggestions that European populations of *P. acuta* have been founded from North America in historic times. Genotype frequencies at allozyme encoding loci yield a negligible coefficient of inbreeding attributable to the continent level of subdivision ($F_{CT}$ = -0.027). European populations are not less heterozygous than their putative sources in America, nor do they seem to have lost alleles. Population F and I are more similar to each other than either is to any of the American populations, missing the common American allele $Lap^{103}$ and sharing the rare allele $Pgm2^{110}$ not detected elsewhere. The sequence data yield a similarly negligible fixation index attributable to the continent level of organization ($F_{CT}$ = -0.065), neither European population showing any evidence of reduction in haplotype diversity (Table 1). Thus any artificial introduction of *P. acuta* into Europe would seem to have involved a large number of colonists, and a specific American source does not immediately present itself. A model hypothesizing multiple colonization events, perhaps from source(s) in the southern Gulf regions of the United States (Anderson 2003), would seem fertile ground for future inquiry.

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663 FIGURE LEGEND

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665 Figure 1: Neighbor-joining analysis of concatenated mtDNA sequences (16S + CO1) from six
666 populations of *Physa acuta* (F, I, P, C, N, D), one population of *P. hendersoni* (Y) and one
667 population of *P. gyrina* (G). Asterisks at the nodes indicate the percent of 10,000 bootstrap
668 replicates supporting each, one indicating greater than 50% and two greater than 90%.

Table 1. Sample sizes (N), numbers of unique haplotypes, and range of genetic distances (uncorrected p) in six populations of *Physa acuta* based on 16S sequence alone (with loops, 581 bp counting spaces), CO1 sequence alone (635 bp used), and the two genes concatenated with16S loops deleted (1145 bp).

| | Population | D | N | P | C | F | I | Total |
|---|---|---|---|---|---|---|---|---|
| 16S alone | N (haplotypes) | 21 (17) | 21 (12) | 17 (14) | 20 (14) | 22 (11) | 19 (10) | 120 (77) |
| | Mean # Pairwise diff. | 16.71 ± 7.75 | 7.41 ± 3.61 | 9.28 ± 4.49 | 3.33 ± 1.78 | 5.68 ± 2.83 | 27.21 ± 12.51 | 35.18 ± 15.42 |
| | genetic distance | 0% - 13.1% | 0% - 2.7% | 0% - 3.5% | 0% - 1.24% | 0% - 3.7% | 0% - 12.56% | 0% - 15.69% |
| | Nuc. diversity | 0.032 ± 0.017 | 0.017 ± 0.009 | 0.018 ± 0.010 | 0.006 ± 0.004 | 0.011 ± 0.006 | 0.072 ± 0.037 | 0.066 ± 0.032 |
| CO1 alone | N (haplotypes) | 15 (15) | 18 (17) | 13 (11) | 15 (7) | 14 (10) | 19 (11) | 94 (70) |
| | Mean #Pairwise diff. | 20.19 ± 9.46 | 14.76 ± 6.92 | 7.99 ± 3.97 | 8.86 ± 4.33 | 2.26 ± 1.32 | 17.57 ± 8.17 | 28.25 ± 12.47 |
| | genetic distance | 0.47%-13.7% | 0% - 5.0% | 0% - 2.42% | 0% - 4.25% | 0% - 3.42% | 0% - 12.0% | 0% - 13.7% |
| | Nuc. diversity | 0.035 ± 0.018 | 0.024 ± 0.012 | 0.013 ± 0.007 | 0.014 ± 0.008 | 0.004 ± 0.003 | 0.034 ±0.017 | 0.043 ± 0.021 |
| 16S + CO1 | N (haplotypes) | 15 (15) | 18 (18) | 12 (10) | 14 (11) | 14 (14) | 14 (10) | 87 (79) |
| | Mean # Pairwise diff. | 35.41 ± 16.35 | 19.67 ± 9.13 | 13.54 ± 6.55 | 11.92 ± 5.56 | 7.18 ± 3.58 | 33.57 ± 15.58 | 45.75 ± 20.15 |
| | genetic distance | 0.35% - 13.3% | 0.18% - 3.6% | 0% - 2.53% | 0% - 2.81% | 0.09% - 3.28% | 0% -10.3% | 0% - 13.3% |
| | Nuc. diversity | 0.033 ± 0.017 | 0.019 ± 0.010 | 0.012 ± 0.006 | 0.010 ± 0.006 | 0.007 ± 0.004 | 0.037 ± 0.019 | 0.042 ± 0.020 |

Table 2. Analysis of molecular variance among six populations of *P. acuta* from Europe and North America, based on the concatenated sequences of two genes, CO1 + 16s (loops deleted).

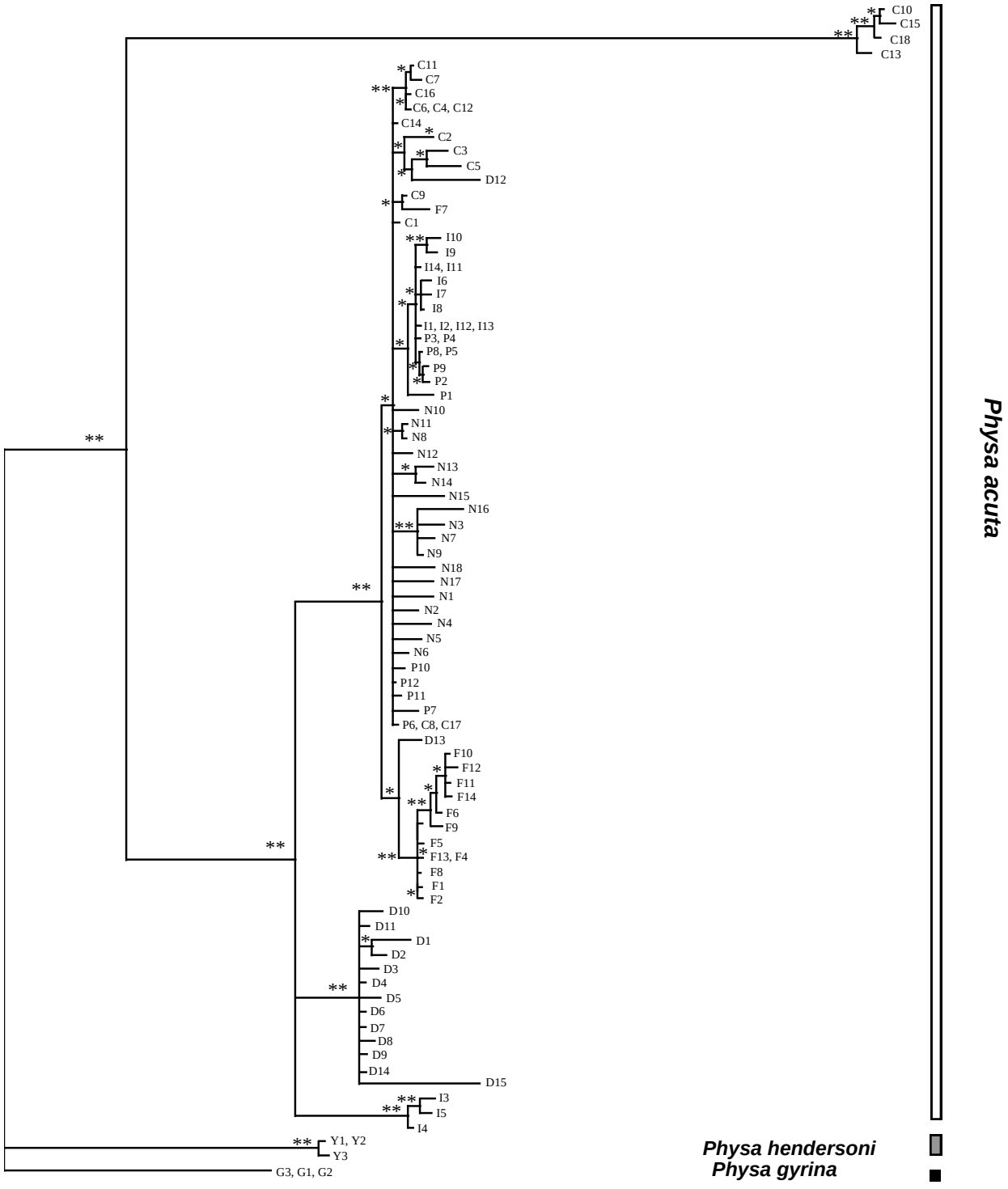
| Source of variation | df | SS | Var | % | p |
|---|---|---|---|---|---|
| Among continents | 1 | 161.441 | -1.50811 | -6.47 | 0.07331 |
| Among pops within continent | 4 | 891.230 | 14.47863 | 62.13 | 0.00000 |
| Within populations | 82 | 847.352 | 10.33356 | 44.34 | 0.00000 |
| Total | 87 | 1900.023 | 23.30408 | | |

Table 3. Allele frequencies at seven allozyme loci and mean (direct-count) heterozygosity over six populations of *P. acuta*, one population of *P. gyrina* (G) and one population of *P. hendersoni* (Y).

| Locus | G | Y | D | N | P | C | F | I |
|---|---|---|---|---|---|---|---|---|
| EST3 | | | | | | | | |
| 105 | 0.000 | 0.000 | 0.842 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 100 | 0.000 | 0.000 | 0.158 | 0.767 | 0.531 | 0.743 | 0.698 | 0.618 |
| 99 | 0.456 | 0.397 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 96 | 0.000 | 0.603 | 0.000 | 0.233 | 0.469 | 0.257 | 0.302 | 0.382 |
| 90 | 0.544 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| LAP1 | | | | | | | | |
| 103 | 0.000 | 0.474 | 1.000 | 0.646 | 0.557 | 0.500 | 0.000 | 0.000 |
| 100 | 0.000 | 0.526 | 0.000 | 0.354 | 0.443 | 0.500 | 1.000 | 1.000 |
| 95 | 0.879 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 92 | 0.121 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 6PGD | | | | | | | | |
| 100 | 0.000 | 0.936 | 1.000 | 1.000 | 0.881 | 1.000 | 0.938 | 1.000 |
| 95 | 0.000 | 0.038 | 0.000 | 0.000 | 0.119 | 0.000 | 0.063 | 0.000 |
| 92 | 0.691 | 0.026 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 88 | 0.309 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| EST6 | | | | | | | | |
| 104 | 0.000 | 0.000 | 0.000 | 1.000 | 0.739 | 0.206 | 0.000 | 0.463 |
| 102 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.006 | 0.329 | 0.000 |
| 100 | 0.000 | 1.000 | 0.000 | 0.000 | 0.261 | 0.789 | 0.671 | 0.537 |
| 92 | 0.000 | 0.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 88 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| PGM2 | | | | | | | | |
| 113 | 0.265 | 0.718 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 110 | 0.735 | 0.115 | 0.000 | 0.000 | 0.000 | 0.000 | 0.023 | 0.065 |
| 103 | 0.000 | 0.154 | 1.000 | 0.000 | 0.053 | 0.013 | 0.000 | 0.000 |
| 100 | 0.000 | 0.013 | 0.000 | 1.000 | 0.947 | 0.987 | 0.977 | 0.935 |
| ISDH | | | | | | | | |
| 104 | 0.000 | 0.013 | 0.423 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 100 | 0.000 | 0.077 | 0.577 | 0.500 | 0.560 | 0.943 | 1.000 | 0.891 |
| 97 | 0.000 | 0.000 | 0.000 | 0.000 | 0.048 | 0.000 | 0.000 | 0.000 |
| 94 | 0.000 | 0.897 | 0.000 | 0.500 | 0.393 | 0.057 | 0.000 | 0.109 |
| 90 | 1.000 | 0.013 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| GPI1 | | | | | | | | |
| 100 | 0.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| 94 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| H | 0.186 | 0.199 | 0.107 | 0.148 | 0.258 | 0.147 | 0.143 | 0.212 |
| (s.e.) | (0.072) | (0.064) | (0.071) | (0.075) | (0.062) | (0.061) | (0.073) | (0.097) |

Table 4. Matrix of Nei (1978) unbiased genetic identities (above diagonal) and genetic distances below the diagonal.

| Population | G | Y | D | N | P | C | F | I |
|---|---|---|---|---|---|---|---|---|
| G | - | 0.091 | 0.000 | 0.000 | 0.000 | 0.000 | 0.003 | 0.009 |
| Y | 2.397 | - | 0.457 | 0.563 | 0.658 | 0.644 | 0.599 | 0.625 |
| D | **** | 0.783 | - | 0.514 | 0.532 | 0.535 | 0.430 | 0.438 |
| N | **** | 0.574 | 0.665 | - | 0.978 | 0.852 | 0.753 | 0.848 |
| P | **** | 0.419 | 0.631 | 0.022 | - | 0.914 | 0.835 | 0.910 |
| C | **** | 0.440 | 0.626 | 0.160 | 0.090 | - | 0.944 | 0.942 |
| F | 5.809 | 0.512 | 0.843 | 0.284 | 0.180 | 0.058 | - | 0.969 |
| I | 4.762 | 0.471 | 0.826 | 0.165 | 0.094 | 0.059 | 0.031 | - |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G3, G1, G2
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