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Physella acuta: atypical mitochondrial gene order among panpulmonates (Gastropoda)

Journey R. Nolan, Ulfar Bergthorsson and Coen M. Adema

Center for Evolutionary and Theoretical Immunology (CETI), Department of Biology MSC03 2020, University of New Mexico, 1 University Blvd NE, Albuquerque, NM 87131, USA

Correspondence: C. M. Adema; e-mail: coenadem@unm.edu

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ABSTRACT

Mitochondrial (mt) sequences are frequently used for phylogenetic reconstruction and for identification of species of molluscs. This study expands the phylogenetic range of Hygrophila (Panpulmonata) for which such sequence data are available by characterizing the full mt genome of the invasive freshwater snail *Physella acuta* (Physidae). The mt genome sequences of two *P. acuta* isolates from Stubblefield Lake, New Mexico, USA, differed in length (14,490 *vs* 14,314 bp) and showed 11.49% sequence divergence, whereas ITS1 and ITS2 sequences from the nuclear genome differed by 1.75%. The mt gene order of *P. acuta* (*cox1*, *P*, *nad6*, *nad5*, *nad1*, *D*, *F*, *cox2*, *Y*, *W*, *nad4L*, *C*, *Q*, *atp6*, *R*, *E*, *rrnS*, *M*, *T*, *cox3*, *I*, *nad2*, *K*, *V*, *rrnL*, *L1*, *A*, *cytb*, *G*, *H*, *L2*, *atp8*, *N*, *nad2*, *S1*, *S2*, *nad4*) differs considerably from the relatively conserved gene order within Panpulmonata. Phylogenetic trees show that the 13 protein-encoding mt gene sequences (equivalent codons) of *P. acuta* group according to gastropod phylogeny, yet branch lengths and dN/dS ratios for *P. acuta* indicate elevated amino acid substitutions relative to other gastropods. This study indicates that mt sequences of *P. acuta* are phylogenetically informative despite a considerable intraspecific divergence and the atypical gene order in its mt genome.

INTRODUCTION

Mitochondrial (mt) gene sequences are commonly used to reconstruct phylogenetic relationships (Boore, 1999; Valles & Boore, 2006), but obtaining entire mitochondrial genomes provides greater amounts of sequences for analysis, identification of mt gene order and discovery of novel mt gene rearrangements. Comparative mitogenomic analyses can inform on animal phylogeny (Rokas & Holland, 2000; Knudsen *et al*., 2006; Jex *et al*., 2010; Kayal *et al*., 2013).

The classes of Mollusca display diverse sets of mt gene orders (Kurabayashi & Ueshima, 2000; Boore, Medina & Rosenberg, 2004; Grande, Templado & Zardoya, 2008). Within the Gastropoda, a generally standard order of mt genes has been recorded in Panpulmonata (Knudsen *et al*., 2006; White *et al*., 2011), a clade established by Jörger *et al*. (2010). Still, the mt genomes of Panpulmonata are no exception to frequent, but minor, gene rearrangements that mainly involve modest numbers of tRNA genes but occasionally also single protein-encoding genes, as seen in *Cepea nemoralis* (Terrett, Miles & Thomas, 1996), *Pyramidella dolabrata* (Grande *et al*., 2008), *Siphonaria gigas* (White *et al*., 2011) and *S. pectinata* (Grande *et al*., 2008).

Our current insights are restricted by the incomplete phylogenetic coverage that is provided by the 24 panpulmonate species from which mt genomes have been sequenced completely. Panpulmonata contains the medically important clade Hygrophila; many of these freshwater snails are intermediate host for flatworm parasites and transmit infectious diseases of human and veterinary importance such as fascioliasis (Mas-Coma, Valero & Bargues, 2009), clonorchiasis and paragonimiasis (Rozendaal, 1997), cercarial dermatitis and schistosomiasis (Morgan *et al*., 2002). Based on phylogenetic analysis of 16S, 18S and CO1 mt gene sequences, Hygrophila was divided into five families: Acroloxidae, Chilinoidae, Planorbidae, Lymnaeidae and Physidae (Dayrat *et al*., 2011). Perhaps because the mt genomes of freshwater panpulmonates are considered difficult to sequence (White *et al*., 2011), so far complete mt genomes are available only for two families of Hygrophila: Planorbidae [*Biomphalaria glabrata* (DeJong, Emery & Adema, 2004) and *B. tenagophila* (Jannotti-Passos *et al*., 2010)] and Lymnaeidae [*Radix balthica* (Feldmeyer, Hoffmeier & Pfenninger, 2010) and *Galba pervia* (Liu *et al*., 2012)]. No mt genome sequences have previously been available for the family Physidae.

Physids are the most abundant and diverse freshwater gastropods native to North America and due to their invasive nature occur throughout the world (Burch, 1989). The phylogeny of Physidae is complex but 16S and COI mt sequences combined with morphological features have been used to reorganize taxonomy of North American physids (Wethington & Lydeard, 2007). *Physella acuta* (Draparnaud, 1805), frequently designated *Physa acuta*, is a widely used model snail that is widely distributed, readily obtainable and can be maintained with ease in the

Table 1. Primers used to characterize the mitochondrial genomes of *Physella acuta*.

		Primer (5′–3′)	3′ Position of primer (A/B)
	1	Pa16SF	TAAAGTGGTATTAGATCTGACGA	10780/10598
		*H3080	ACGTGATCTGAGTTCAGACCGG	10915/10733
		PaCYBF	GGAGATCACATACTTGCCAAGACC	11200/11017
	(7)	PaCYBR	TCAAAAGATCTGGCGATATTAGCC	11296/11114
2		*ATP*8JF	AATTCCATAAGTGGGGCTGAG′	12610/12431
		ND3JR	TCTTGAAAGTGTCGTGATCCT	13040/12608
		*ATP*8JFC	CCTCTTGATATACCTCTGGATCG	13080/12902
		ND4JR (B)	ATGTCCAACTGACGAATACGC	13986/13810
		*LCO1490	GGTCAACAAATCATAAAGATATTGG	38
		A_CO1JRC	AAACCTGTACCGACCAATCC	90
		B_CO1JRC	CAAAAGCATGTGCTGTAACG	159
	3	PaCO1F	GTTTGATCGGTGTTAATTACTGCA	564
		*LCO2198	TAAACTTCAGGGTGACCAAAAAATCA	694
		CO1JFC	CGAGCTTATTTTACAGCAGCAAC′	911
		ND5JRC	GACGTGATTCCTAATCCATCTCAC	2371
4		ND5JF	TAAGGCAATGCTTTTCATGG	2939
		ND5JR	GGAATACCCATTAATGAAAGTCCAC	3042
		ND5JFC	ATCGGTTCCGTAAACACGTC	3249
		CO2JRC	CCTCCTGAATAGGTGATGCTG	4701/4699
	5	PaCO2F	AACAAGTGCTGACGTATTGCATGC	5127/5125
		CO2JR2	CAATGACAGGCACTAATATCTGC	5562/5367
		A_ND4LJFC	TTTGGTGGCAGATATGTAGTGC	5576/–
		B_ND4LJFC	GCCCTGGGACTGACCTTG	–/5698
		A_ATP6JF2	AAGCTCAAATCTTTTTGTGCAAC	6064/5869
		12SJRC	GTGGGGCACAAATGTAGGAC	7428/7237
6		CO3JF	GTTATGGGCCCAATAGCTTC	7679/7469
		CO3JR	ACCACGTTGGATTCTTAGCC′	7855/7666
		CO3JFC	CCTCAATGGCATGATGAGC	8127/7685
		ND2JRC	GACTTTCGGGTAAAACAACAGG	9381/9195
	7	ND2JF	CCTGTTGTTTTACCCGAAAGTC	9402/9216
		16SJR2	ATACTTTTCCCCGCTATCCAG	10051/9863
		N2G16SJFC	CCTTTCAAATTTTGTGATAGCTG	10053/9865
		*L2510	CGCCTGTTTATCAAAAACAT	10418/10232

Lines to the left of the primers delineate the seven overlapping long distance PCR amplicons that were cloned and sequenced to confirm data obtained by direct sequencing. Amplicons 1 and 7 overlap due to the circular nature of the mt genome, (7) indicates the end of the 7th fragment. Asterisks indicate conserved 16S and COI primers for species identification (Wethington & Lydeard, 2007).

laboratory. This species serves as an aquatic biomarker due to its ability to live in polluted water (Sánchez-Argüello, Fernández & Tarazona, 2009; Lee *et al*., 2011), it has high salinity thresholds (Kefford & Nugegoda, 2005) and it is used in population and mating studies (Bousset *et al*., 2004; Dillon, Wethington & Lydeard, 2011). As an invasive species, *P. acuta* has been studied for competitiveness with indigenous gastropod fauna (Madsen & Frandsen, 1989; Albrecht *et al*., 2009). Here, we characterize the mt genome of *P. acuta*.

In this study, 16S and COI mt sequences (Wethington & Lydeard, 2007) are used for species identification of laboratory maintained physid snails. In addition, sequences from the nuclear genome, internal transcribed spacer (ITS)1 and ITS2, are also employed. These ITS sequences are often used for species identifications at lower taxonomic levels (Armbruster & Korte, 2006), including species identification within Hygrophila (DeJong *et al*., 2001; Correa *et al*., 2010). The mt genomes from two isolates of *P. acuta* (A and B) are characterized and compared. The mt genes and gene order from these physid snails are compared with those of other panpulmonates. Finally we perform a rate analysis and determine dN/dS ratios of mt protein-encoding genes of *P. acuta* to investigate the rate of genome evolution relative to other panpulmonates.

MATERIAL AND METHODS

Snail isolates, DNA extraction and species identification

In 2010, freshwater panpulmonate snails, morphologically identified as physids (sinistral shells, digitations on mantle collar; Paraense & Pointier, 2003) were collected from Stubblefield Lake in northern New Mexico (USA) and maintained in aquariums at room temperature. Separate lines of laboratory-cultured physid snails were initiated with hatchlings from recently deposited single egg masses that were isolated in different tanks. This approach was used to separate morphologically similar yet genetically distinct lineages (Wethington & Lydeard, 2007) and to avoid pre-existing (trematode) parasite infections in the parental snails that were collected from the field. Two separate lines of physids were established, designated as isolates A and B.

Total DNA was extracted from whole body tissues from individual snails (4–6 mm shell length) using a cetyltrimethylammonium bromide (CTAB)-based method (Winnepenninckx, Backeljau & De Wachter, 1993). For taxonomic identification, PCR (AmpliTaq Gold, Applied Biosystems) was performed to amplify sequences fragments from the phylogenetically informative mt genes 16S (Palumbi *et al*., 1991) and COI (Folmer *et al*.,

1994) as described by Wethington & Lydeard (2007). Primers are listed in Table 1. The complete nuclear ITS1 and ITS2 regions were amplified using the following primers: ITS1 5′TAACAAGGTTTCCGTATGTGAA3′ (Armbruster & Bernhard, 2000) and ITS2R 5′GGTTTCACGTACTCTTGAAC3′ (provided by J. Nekola, modified from that published by Wade & Mordan, 2000). Termini of ITS regions were assigned by identifying flanking ribosomal DNA gene boundaries according to DeJong *et al.* (2001). Thermal cycling consisted of 10 min at 94°C (initial denaturation), 25 cycles of 30 s at 94°C, 30 s at primer annealing temperatures (50°C for 16S and COI, 48°C for ITS regions) , 1 min at 72°C, and 7 min 72°C final extension. Amplicons were purified (QIAquick PCR purification Kit, Qiagen) and sequenced directly on both strands (Big Dye 3.1, Applied Biosystems). Extension products were read on an ABI 3130 automated DNA sequencer. Sequences were edited by eye and assembled into contigs using Sequencher v. 5.0 (Gene Codes Corporation). The sequences were compared with the GenBank database using BLAST (Altschul *et al.*, 1997) for gene identification. Phylogenetic analyses of COI and 16S sequences from the *P. acuta* isolates were performed using neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) (Gamma distribution + invariant sites) to place the experimentally-obtained nucleotide sequences in the context of separate pre-existing COI- and 16S-based phylogenies of Physidae, which also included members of Lymnaeidae and Planorbidae as outgroups (NCBI popset: 164430598 and NCBI popset: 164430551, respectively; Wethington & Lydeard, 2007) with 1,000 replicates using MEGA v. 5.05 (Tamura *et al.*, 2011).

The uncorrected p-distances (proportion of nucleotide sites at which sequences differ; Nei & Kumar, 2000) were calculated for each of 16S, COI, ITS1 and ITS2 sequences and the full length mt genome from the two isolates of *P. acuta*, and for several publicly-available sequences to determine and compare ranges of intra- and interspecific sequence differences. Intraspecific differences were determined for 16S sequences of *P. acuta* (NCBI popset: 164430551; Wethington & Lydeard, 2007) and *Biomphalaria glabrata* (NCBI popset: 15717799; DeJong *et al.*, 2001), COI sequences from *P. acuta* (Albrecht *et al.*, 2009), and ITS1 and ITS2 sequences from 12 species of *Biomphalaria* (NCBI popset: 15717841; DeJong *et al.*, 2001). Interspecific differences among entire mt genomes or selected genes from four genera were determined for *Aplysia: A. californica* (GenBank: NC005827; Knudsen *et al.*, 2006); *A. dactlyomela* (GenBank: NC015088; Medina *et al.*, 2011) and *A. vaccaria* (GenBank: DQ991928; Medina *et al.*, 2011); *Biomphalaria: B. glabrata* (GenBank: NC005439; DeJong *et al.*, 2004) and *B. tenagophila* (GenBank: NC010220; Jannotti-Passos *et al.*, 2010); *Onchidella: O. borealis* (GenBank: DQ991936; Medina *et al.*, 2011) and *O. celtica* (GenBank: NC012376; Grande *et al.*, 2008); and *Siphonaria: S. gigas* (GenBank: NC016188; White *et al.*, 2011) and *S. pectinata* (GenBank: NC012383; Grande *et al.*, 2008).

Full mitochondrial genome sequencing

Complete mt genomes were characterized from single individual snails, one each from *P. acuta* isolates A and B. PCR primers (Table 1) were designed and optimized using Primer3 (Rozen & Skaletsky, 2000) to target conserved regions of mt genes that were identified in alignments of previously reported complete mt genome sequences from panpulmonate species and EST data available from GenBank (Lee *et al.*, 2011; White *et al.*, 2011). High fidelity, long distance (LD)-PCR (Advantage Genomic LA Polymerase Mix, Clontech) was used to generate overlapping amplicons that encompassed the complete mt genome. Amplicons were sequenced directly by primer walking (see above) at double coverage or higher. Chromatograms were edited by eye and assembled into contigs using Sequencher v. 5.0. Once mt genome sequences of isolates A and B were characterized completely, primers listed in Table 1 were used to generate seven overlapping PCR fragments (range 1931–2624 bp) from the same original genomic DNA templates, which completely covered the mt genomes. High fidelity LD-PCR amplicons were cloned (TOPO TA-cloning, Invitrogen) and sequenced completely to confirm the mt sequence data.

Annotation and comparison of P. acuta mitochondrial genomes

BLAST was used to identify protein-encoding and rRNA mt genes of *P. acuta* isolates A and B. Gene termini were designated based on open reading frame (ORF) analyses to minimize overlap with adjacent genes, considering alternative start and stop codons, and finally checking predictions against RNA-SEQ data from *P. acuta* (J.R. Nolan & C.M. Adema, unpubl.). The mapping of tRNA genes was based on identification of anticodons surrounded by sequences that formed secondary structures, similar to DeJong *et al.* (2004). The predicted secondary structures of tRNAs were visualized with RNAviz2 (De Rijk, Wuyts & De Wachter, 2003). Codon usage was determined using MEGA v. 5.2 (Tamura *et al.*, 2011). To predict the location of the potential origin of replication (POR), the following were considered: (1) noncoding regions >40 bp in length containing high, localized AT richness and predictive 5′ TATA sequence repeats as seen in *Drosophila* (Kilpert & Podsiadlowski, 2006); (2) regions with high GC skew [(G-C)/(G + C)] (Xia, 2012) using window size 2500 nt and step size 72 nt, (CGview; Stothard & Wishart, 2005); (3) POR locations as hypothesized for other panpulmonates (Grande *et al.*, 2008; White *et al.*, 2011). Mt genomes were depicted graphically using Artemis (Rutherford *et al.*, 2000). The mt genomic sequences from *P. acuta* isolates A and B were compared for length, indels, nucleotide content and predicted amino acid composition using Sequencher v. 5.0.

Mitochondrial gene order: P. acuta vs other panpulmonates

Starting with *cox1*, the order of mt genes recorded from *P. acuta* isolates A and B was depicted in linear fashion and aligned with mt genomes of basal and derived Panpulmonata, as inferred from 18S, 28S, 16S and CO1 sequence data (Jörger *et al.*, 2010): *S. pectinata* (basal) (GenBank: NC012383; Grande *et al.*, 2008); *Salinator rhamphidia* (Amphiboloidea; GenBank: NC016185; White *et al.*, 2011); *Ovatella vulcani* (GenBank: NC016175) and *Trimusculus reticulatus* (GenBank: NC016193) (both Ellobiidae; White *et al.*, 2011); *Rhopalocaulis grandidieri* (Veronicellidae; GenBank: NC016183; White *et al.*, 2011) and *O. celtica* (Onchidiidae; GenBank: NC012376; Grande *et al.*, 2008) (both Systellomatophora); *Albinaria caerulea* (GenBank: NC001761; Hatzoglou, Rodakis & Lecanidou, 1995) and *C. nemoralis* (GenBank: NC001816; Yamazaki *et al.*, 1997) (both Stylommatophora); *Pyramidella dolabrata* (Pyramidellidae; GenBank: NC012435; Grande *et al.*, 2008); and from members of two sister families of the Physidae within the Hygrophila, *B. glabrata* (Planorbidae; GenBank: NC005439; DeJong *et al.*, 2004) and *Radix balthica* (Lymnaeidae; GenBank: HQ330989; Feldmeyer *et al.*, 2010).

Substitution rates of mitochondrial genomes of P. acuta vs other gastropods

NJ, MP and ML analyses were performed to investigate the phylogenetic relationship of *P. acuta* with other gastropods and to determine branch lengths as a measure of divergence. Complete nucleotide sequences for protein-encoding genes were obtained from Genbank for the panpulmonates listed above. The phylogenetic range was expanded by also including sequences from *Aplysia californica* (GenBank: NC005827; Knudsen *et al.*, 2006), a

euopisthobranch, and basal outgroups *Dendropoma maximum* (GenBank: NC014583; Rawlings *et al*., 2010), *Conus textile* (GenBank: NC008797; Bandyopadhyay *et al*., 2008), *Haliotis rubra* (GenBank: NC005940; Maynard *et al*., 2005), *Nerita melanotragus* (GenBank: GU810158; Castro & Colgan, 2010) and *Lottia digitalis* (GenBank: NC007782; Simison, Lindberg & Boore, 2006). The mt genome sequence of the lymnaeid *R. balthica* was not used because of the low quality of the 454-reads with respect to length of mononucleotide tracts (Feldmeyer *et al*., 2010). The protein-encoding gene sequences of the 16 gastropods and *P. acuta* isolates A and B were individually translated, aligned and cropped by hand to remove highly divergent, nonalignable gap-columns using COBALT (Papadopoulous & Agarwala, 2007) and Bioedit (Hall, 1999). Gene sequences were then concatenated for each gastropod and phylogenetic analyses were performed with MEGA v. 5.05, using integrated utilities for selection of model as best fit for the data. Phylogenetic NJ (Jones-Taylor-Thornton model with gamma distributed rate variation among sites), MP (10 initial trees by random addition) and ML (WAF + F + G, 5 gamma categories) analyses were performed with 1,000 bootstrap replicates.

The relative rate test (Tajima, 1993) was performed in MEGA v. 5.05 to test the mt genomes of *P. acuta* for accelerated nucleotide and amino acid substitution rates relative to *B. glabrata* (with *Pyramidella dolabrata* as an outgroup) using aligned sequences with gaps removed.

The GA-Branch program was used through the Datamonkey portal (Kosakovsky, Pond & Frost, 2005) to identify terminal branches with significantly different dN/dS ratios in the gastropod ML tree. The dN/dS ratios were generated from 12 of the 13 protein-encoding gene sequences from the two isolates of *P. acuta* along with selected gastropods to investigate substitution rates of *P. acuta* compared to other gastropods. Due to short length of alignable codons, *atp8* was excluded in dN/dS analyses. The gastropod species *A. californica*, *C. nemoralis* and *N. melanotragus* appeared to have undergone rate acceleration and were excluded from this analysis. The nucleotide sequences of individual protein-encoding genes were translated, aligned and gap columns were removed to analyse dN/dS ratios for each gene and also for the concatenated gene sequences to identify amino acid substitutions across the mt genomes as a whole.

RESULTS

Species identification

The initial morphology-based identification as physid snails was confirmed when BLAST searches revealed greatest similarities of sequence from both isolates A and B to GenBank entries of ITS1, ITS2, 16S and COI from *Physella acuta*. The ITS1 sequences (A: 495 bp, GenBank: KF316327; B: 497 bp, GenBank: KF316329) differed at 10 nucleotide (nt) positions and ITS2 sequences (A: 301 bp, GenBank: KF316328; B: 302 bp, GenBank: KF316326) differed by 4 nt (for alignments see Supplementary material Tables S1 and S2). The combined ITS regions differed in nt sequence by 1.75% between *P. acuta* A and B. This value falls within the intraspecific divergence for the combined ITS fragments of 12 different *Biomphalaria* species, ranging from 0% (*B. alexandrina*) to 2.70% (*B. glabrata*) (DeJong *et al*., 2001). The mt genome-derived sequences (GenBank accession numbers in following paragraph) from isolates A and B showed a higher divergence. The amplicons from the 16S genes were 496 bp (A) and 500 bp (B) with a 5.38% nt difference evident from the sequence alignment (length 502 bp with gaps). The COI sequence fragments, 655 bp for both isolates, displayed a 4.27% nt difference. The sequences were confirmed from sibling snails of both isolates. Combining both 16S and COI, the total sequence difference was 4.75% over 1151 bp. Based on a threshold of <6% difference in these combined sequences, as defined by Wethington & Lydeard (2007), both isolates are representatives of the species *P. acuta*. This divergence between mt sequences of *P. acuta* A and B is less than the maximum intraspecific divergence calculated at 7.0% for 16S sequences from *B. glabrata* (DeJong *et al*., 2001) and at 11.9% from COI sequences reported for *P. acuta* elsewhere (Albrecht *et al*., 2009). Accordingly, analysis of the COI sequences relative to a previously reported phylogeny of physid snails (Wethington & Lydeard, 2007) placed isolates A and B within the clade of *P. acuta*, with the two isolates representing separate genetic lineages of the species (Fig. 1). Similar results were obtained with 16S sequences (data not shown).

General features of mitochondrial genome of P. acuta

The complete mt genomes of isolates A and B were characterized (A: GenBank: JQ390525; B: GenBank: JQ390526) and while they differed considerably in sequence composition (see below), the following features are held in common. *Physella acuta* has the standard metazoan complement of mt genes consisting of 13 protein-encoding genes, 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes. The genomes have an AT-content of 69.22% for isolate A and 69.69% for isolate B. The mt gene order is as follows: *cox1*, *P*, *nad6*, *nad5*, *nad1*, *D*, *F*, *cox2*, *Y*, *W*, *nad4L*, *C*, *Q*, *atp6*, *R*, *E*, *rrnS*, *M*, *T*, *cox3*, *I*, *nad2*, *K*, *V*, *rrnL*, *L1*, *A*, *cytb*, *G*, *H*, *L2*, *atp8*, *N*, *nad3*, *S2*, *S1* and *nad4* (Fig. 2). The underlined genes are located on the negative strand of the circular genome. Intergenic regions are evident but the genes are generally spaced closely together. The protein-encoding gene *nad4* has an incomplete stop codon (T_ _); inspection of cDNA transcripts confirmed that this stop codon is completed by mRNA polyadenylation (not shown). Several genes overlap partially; *nad5* and *nad1* overlap by 13 bp, *nad4L* and *trnC* by 2 bp, *trnY* and *trnW* by 7 bp, *trnL1* and *trnA* by 4 bp, and finally *trnC* and *trnQ* (two tRNAs that are located on opposite strands) overlap by 6 bp. The location of the potential origin of replication (POR) is predicted in the intergenic region between *cox3* and *trnI*, upstream of *nad2*. This is one of the largest intergenic regions, 45/48 bp with 84.1%/87.5% AT-richness (*P. acuta* isolate A/B, respectively) and contains predictive 5′ TATA sequence repeats. Additionally, this intergenic region is near a change from low to high G/C skew (Fig. 3), as measured using the method of Xia (2012), showing average values over sequence intervals of 2,500 nt along the mt genome (step size 72), and it has been predicted to contain the POR for other panpulmonates (Grande *et al*., 2008; White *et al*., 2011).

Differences between mitochondrial sequences of P. acuta isolates A and B

The mt genomes from isolates A and B of the same species *P. acuta* are dissimilar in both size (14,490 *vs* 14,314 bp) and in sequence content. With the exception of the tRNAs *I*, *M*, and *P*, every other mt gene homologue differed in sequence composition and/or size (Supplementary material Table S3). The intergenic regions range from 1–226 bp in length, with the latter only recorded from isolate A. The nucleotide composition of the mt genome sequence from the two isolates differ by 9.92% (1,416 nt in 14,275 bp), gaps excluded, this value increases to 11.49% (1,670 nt in 14,529 bp) with the inclusion of indel positions.

A total of 37 indels contribute to the size difference of the two mt genomes. A 193 bp indel occurs in the intergenic region between *cox2* and *trnY*; the 3′ coding region of the *cox2* gene of isolate A contains a 39 bp extension followed by a 154 bp addition to the noncoding region between *cox2* and *trnY*. No indels created frame shifts within protein-encoding gene sequences. Further indels contributed one additional amino acid codon

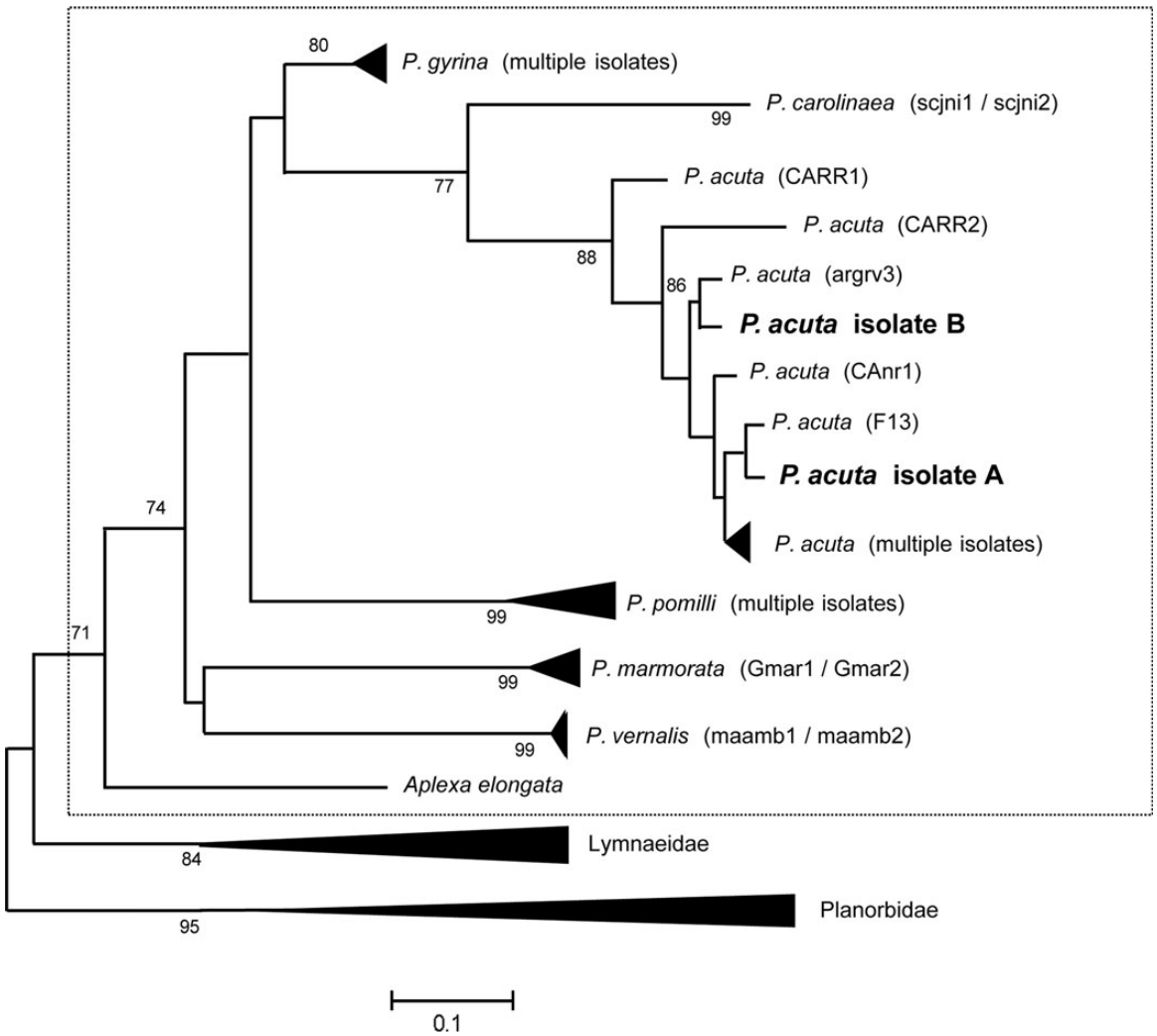

Figure 1. Phylogenetic placement of *Physella acuta* isolates A and B within Physidae. Experimentally derived sequences were incorporated into NCBI popset 164430598 (Wethington & Lydeard, 2007) COI sequences from snails of the family Physidae (boxed) to generate a ML tree; NJ and MP yielded the same results. Original identifiers of strains or isolates of *P. acuta* are indicated in brackets. The outgroup includes sequences from Lymnaeidae and Planorbidae. Isolates A and B, which coexist in Stubblefield Lake, NM (bolded), cluster with different clades of *P. acuta*. The tree has been simplified for clarity, bootstrap values are indicated from 1,000 replicates.

(3 bp) in each of isolate A's *atp6* and *nad1*, an additional one bp in *rrnS* of isolate A, and an additional nine bp in *rrnL* of isolate B. The remaining indels occur in intergenic regions.

Differences in sequence composition occur in 19 of the 30 intergenic regions, both rRNAs, and in 19 of the 22 tRNAs. The nt substitutions between the tRNAs from the isolates A and B typically affect the loops and rarely the stems of the predicted clover-leaf structures (Fig. 4). The protein-encoding genes between the two isolates have a broad range of synonymous and nonsynonymous nt substitutions (Supplementary Material Table S3). Nucleotide sequence differences ranged from 5.26% (*atp8*) to 29.75% (*nad4L*). This affected overall codon usage, with the greatest difference recorded for Leucine (L1): CUA was the dominant codon in isolate A *vs* CUU in isolate B (Supplementary Material Table S4), but this was not significant (CUA $\chi^2 = 0.087$, $P = 0.77$; CUU $\chi^2 = 0.98$, $P = 0.32$). Additionally, (alternative) start codons and stop codons vary between *atp8*, *cox2* and *cytb* gene homologues. The amino acid substitutions ranged from 0.59% (*cox1*) to 25.81% (*nad4L*). With the exception of *cox2* (increased length due to indel), the similarity of protein sequences of *P. acuta* A and B was $\geq$90% due to a majority of synonymous replacements (Supplementary Material Table S3).

The 11.49% overall intraspecific divergence at nt level of complete mt genomes of *P. acuta* A and B exceeds that of two strains of *B. glabrata* (18 of 13,670 nt or 0.13%; uncorrected *p*-distance). This divergence is comparable to interspecific difference from total mt genome sequences among additional species within either the genus *Aplysia* or the genus *Biomphalaria*; however, it did not exceed the interspecific sequence differences from species within the genera *Onchidella* and *Siphonaria*. Regardless of the high intraspecific divergence, *P. acuta* is distinct from other genera. A direct comparison of the *cox1* gene sequences from *P. acuta* isolate A compared with *B. glabrata* (representing the sister taxon), yielded over 20% sequence divergence between genera.

Mitochondrial gene order: P. acuta vs Panpulmonata

The mt gene order of *P. acuta* is novel compared to the rather standard gene order that has been recorded from other panpulmonates (Fig. 5). Despite the rearrangements evident from *P. acuta*, the coding directionality on the positive or negative strand is identical for gene homologues of all the panpulmonates. In addition, several groups of genes that occur adjacent in

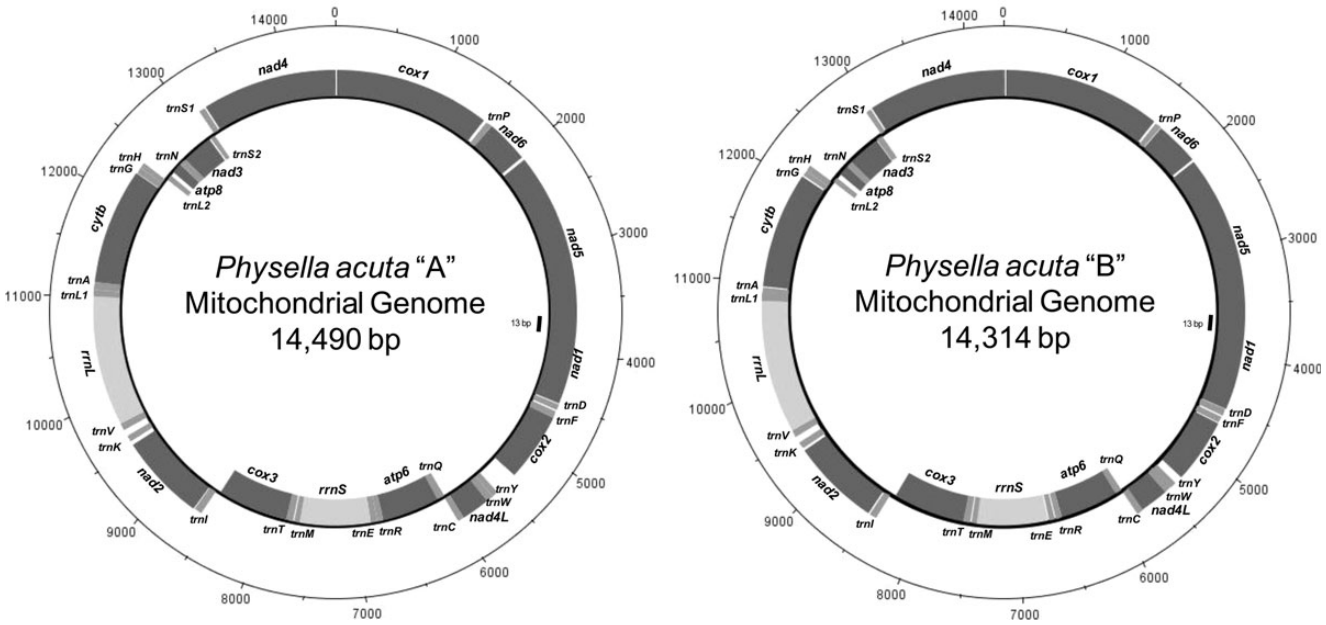

Figure 2. The mitochondrial genomes of *Physella acuta* isolates A and B. The outer circle represents the positive strand, the inner circle the negative strand. Protein-encoding genes are darkened to distinguish from rRNA genes. Bars (with length in bp) indicate location of sequence overlap between protein-encoding genes. Note the size difference of the mt genomes of the two *P. acuta* isolates, especially the indel beginning in *cox2* following the intergenic region upstream of *trnY*.

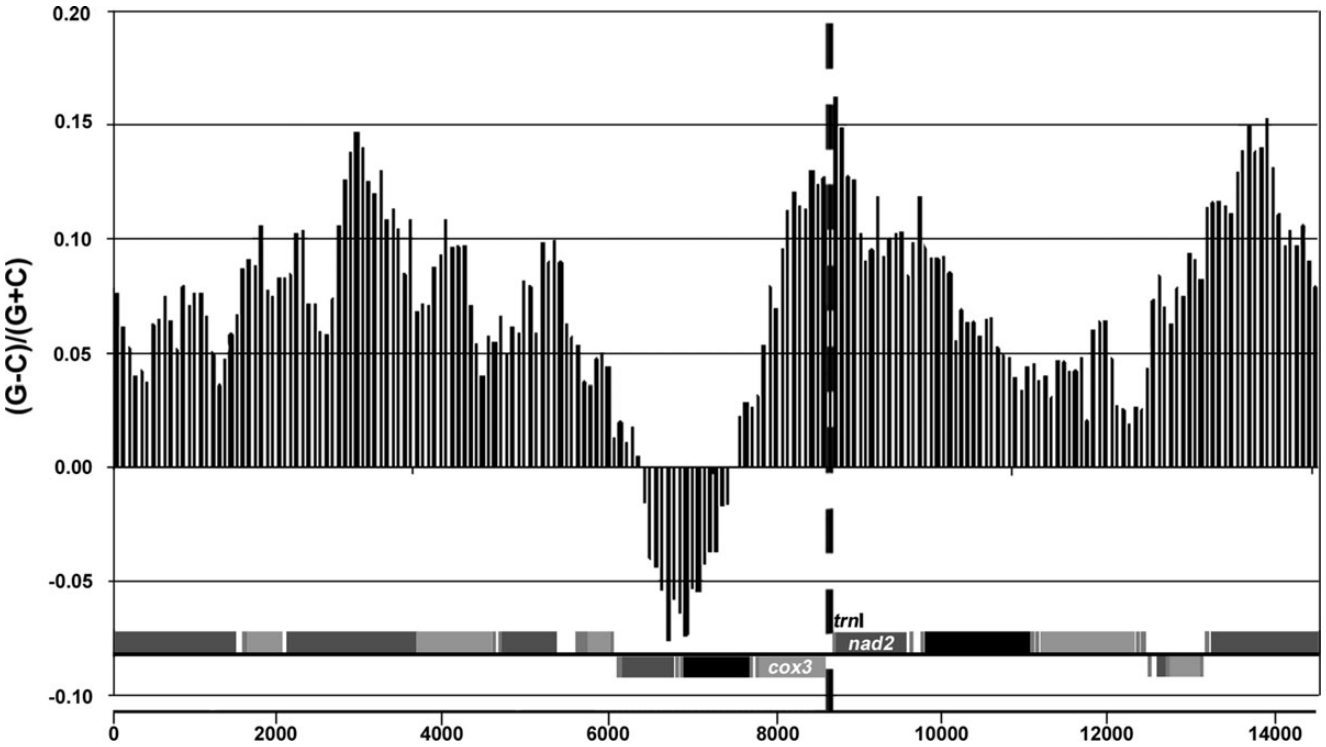

Figure 3. Potential origin of replication (POR), location by GC skew analysis. GC skew [(G − C)/(G + C)] ratios plotted in a bar graph relative to a linear representation of the mt genome of *P. acuta* (isolate A shown). Positive values indicate greater G content and negative values indicate increased C content. The vertical dotted line indicates the predicted location of the POR; note the GC skew maximum at 0.162 that further supports this prediction. This high peak is the origin of the sequence interval (window size 2,500 nt) with the highest GC skew, transitioning from low GC skew upstream (Xia, 2012). Shading of protein-encoding and RNA genes as in Figure 2.

the mt genome of *P. acuta* were designated as gene clusters because identical groups of genes are present (in different order) in the mt genomes of other panpulmonates. The rearranged mt gene order of *P. acuta* may have resulted from processes that have retained several gene clusters (see Supplementary Material Table S5 for a scenario involving segmental duplications and deletions of gene duplicates that may explain the origin of the rearranged gene order in the mt genome of *P. acuta*).

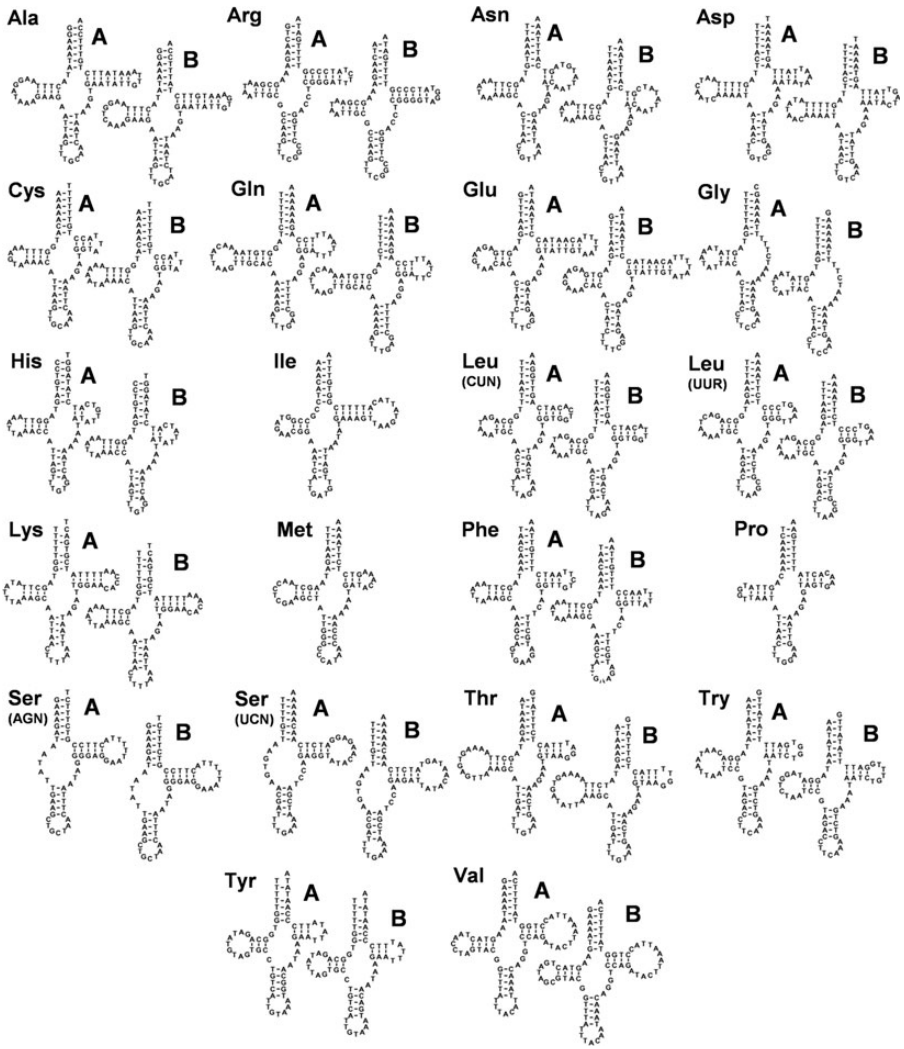

Figure 4. *Physella acuta* isolates A and B: tRNA sequence and structure. Predicted secondary structures of the 22 tRNAs encoded in the mt genomes from *P. acuta* isolates A and B. Only three tRNA genes are identical between isolates A and B. Two graphical representations are shown for all tRNA genes that differ in sequence between isolates A and B. Typically such differences occurred in the loops, not the stems. Three letter codes identify the amino acid anticodon specificity. Irregular tRNAs are Gly, Ser (AGN) and Ser (UCN).

Rate of mutation of mitochondrial genome of P. acuta

The ML tree of the equivalent amino acids predicted from equivalent codons of protein-encoding genes of the mt genomes of *P. acuta* and other selected gastropods is similar to published phylogenies (Fig. 6) (Grande *et al.*, 2008; Klussman-Kolb *et al.*, 2008; Jörger *et al.*, 2010; Dayrat *et al.*, 2011; White *et al.*, 2011). NJ and MP analyses (not shown) yielded similar results. Briefly, the NJ tree showed the hygrophilid species *B. glabrata* and *P. acuta* as adjacent branches (low support), while the MP and ML analyses both showed *B. glabrata* as sister group to *P. acuta* (low support). The long branch lengths for *P. acuta* relative to most other clades (ML), especially close phylogenetic neighbours, is indicative of a higher substitution rate in the mt genomes of *P. acuta*.

The relative rate analysis showed a highly significant acceleration in both nt (not shown) and amino acid substitutions in the mt genomes of *P. acuta* relative to *B. glabrata* (isolate A $\chi^2 = 38.01$, isolate B $\chi^2 = 30.82$, $P < 0.000001$ for each).

The dN/dS ratios for the terminal branches (Table 2) from the ML tree across the protein-encoding genes identified a significant increase of amino acid substitutions in *P. acuta* (0.091) as compared to other gastropods (0.019). Increased dN/dS values for individual genes were recorded for *cox2*, *nad1*, *nad2*, *nad4*, *nad5*, and (isolate B only) *nad6*, but not all were significant (see Table 2). The remaining protein-encoding genes had equivalent dN/dS ratios relative to other gastropods. Note that the *cox1* of isolate A was the only gene with a lower dN/dS ratio as compared to other gastropods. Gene relocations resulting from putative gene rearrangements did not appear to associate with altered dN/dS ratios of particular genes of *P. acuta* as compared to other gastropods (Table 2).

DISCUSSION

The characterization of the mt genome of *P. acuta* revealed (1) considerable intraspecific differences in length and sequence composition, (2) a novel gene order that is unique among panpulmonates and (3) elevated substitution rates in protein-encoding genes compared with mt genomes of other gastropods.

The sequence data (ITS1, ITS2, 16S and COI) obtained from the physid snails collected from Stubblefield Lake identified isolate A and B as the same species, *Physella acuta*. The isolate-specific differences between the sequences that were analysed fell within the ranges of considerable intraspecific divergence that are routinely recorded from phylogenetic studies that employ such genes of other snail species (Thomaz, Guiller & Clarke, 1996; Stothard & Rollinson, 1997; DeJong *et al.*, 2001;

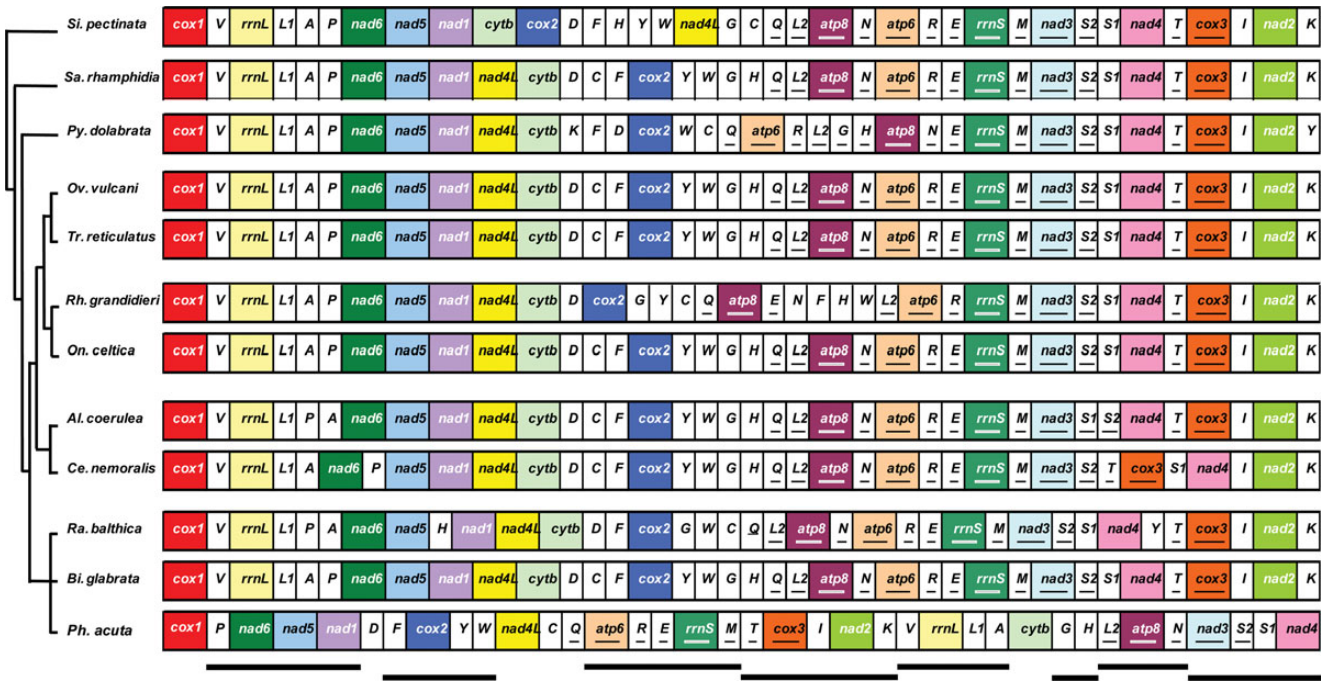

Figure 5. Linear alignment of mt gene order of *Physella acuta* compared with other panpulmonate gastropods. Phylogenetic relationships shown are based on analysis of 18S, 28S, 16S and COI sequences (Jörger *et al*., 2010). Protein-encoding and rRNA genes are colour coded to emphasize patterns and gene rearrangements. Single letters designate tRNA genes. The genes encoded on the negative strand (underlined) are the same for all species shown. Bold lines under the alignment delineate clusters of genes with the same internal order for the majority of the panpulmonates. Note that gene rearrangements among the panpulmonates are modest and rarely affect protein-encoding gene orders, with the exception of *P. acuta*. Alignment is not to scale.

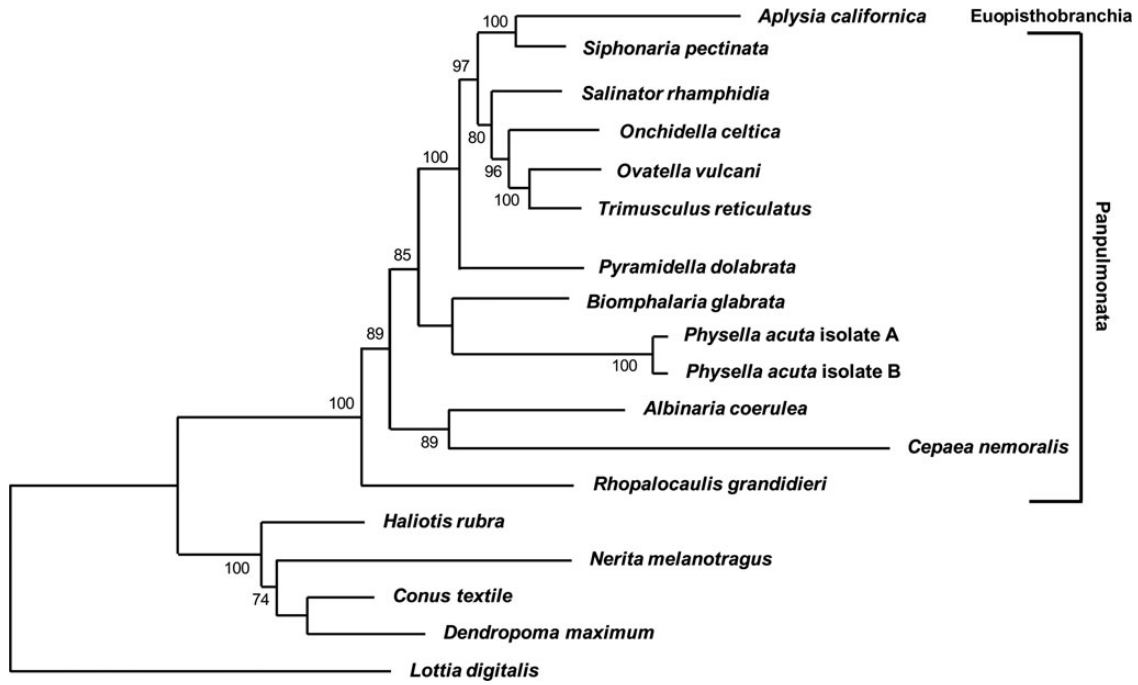

Figure 6. Phylogenetic analysis of selected gastropods. Representative gastropods with fully characterized mt genomes were selected to compare protein-encoding genes with those of *Physella acuta*. Optimized alignments of amino acid sequences of all 13 protein-encoding genes were concatenated for ML analysis (1,000 bootstrap replicates). Longer branch lengths indicate increased mutation rates of amino acid sequences across the mt genome. Note the relatively long branch of the *Physella* clade.

Dillon & Frankis, 2004; Armbruster & Bernhard, 2000; Nekola, Coles & Bergthorsson, 2009; Albrechts *et al*., 2009; Wethington, Wise & Dillon, 2009).

For *P. acuta*, the levels of intraspecific divergence were different for the nuclear ITS sequences (>98% identity) *vs* the mitochondrial 16S and COI sequences (95.25% identity).

Table 2. Nonsynonymous per synonymous (dN/dS) substitution ratios, comparing *Physella acuta* with other gastropods (see Material and Methods).

Gene sequence	dN/dS ratio	
	Other gastropods	*P. acuta* A/B
Concatenated gene set*	0.019	0.091
cox1	0.011	0.006/0.011
nad6	0.101	0.101/0.239
*nad5**	0.016	0.091/0.074
*nad1**	0.026	0.122
cox2	0.042	0.076
nad4L	0.049	
atp6	0.030	
cox3	0.022	
*nad2**	0.033	0.216
cytb	0.035	
atp8	n.d.	
nad3	0.082	
nad4	0.059	0.139

The dN/dS ratios were calculated for individual and concatenated mt protein-encoding gene sequences with the exception of *atp8*. Single ratios indicate no difference between other gastropods and *P. acuta* isolates. Different ratios from isolates of *P. acuta* are separated by a slash; n.d. indicates not done. Genes with significantly different ($P < 0.001$) dN/dS ratio between the *P. acuta* isolates and the other gastropods are indicated by an asterisk.

Differences in 16S and COI gene sequences between the two isolates did not exceed the 6% difference suggested to delineate separate species of *Physella* (Wethington & Lydeard, 2007). Additionally, phylogenetic reconstruction placed isolates A and B within the *P. acuta* clade, but as separate genetic lineages (Fig. 1). The characterization of the complete mt genomes revealed additional extensive differences in sequence and length that further increased the mt nucleotide divergence between *P. acuta* A and B to 11.49%. Based upon the limited number of reports available for such comparison of complete mt genomes, this level exceeds the intraspecific divergence of *B. glabrata* (DeJong *et al*., 2004) and it is more within the range of interspecific divergence within the genera *Biomphalaria* and *Aplysia* (DeJong *et al*., 2004; Knudsen *et al*., 2006; Jannotti-Passos *et al*., 2010; Medina *et al*., 2011). The observation in one gastropod species of minimal intraspecific differences in nuclear sequences combined with elevated divergence of mt sequences is not novel. Additional to *P. acuta*, another instance was reported for the slug *Arion subfuscus* (Stylommatophora) with mean pairwise sequence divergence of 21% for 16S and 0.3% for ITS1 (Pinceel, Jordaens & Backeljau, 2005). Dramatic intraspecific differences occur in some bivalve molluscs where doubly uniparental inheritance (DUI) of maternally (F genome) and paternally (M genome) transmitted mitochondrial genomes differ in size, gene order and sequence (Doucet-Beaupré *et al*., 2010). However, this does not apply here; *P. acuta* belongs to a different molluscan class and is a simultaneous hermaphrodite. Thomaz *et al*. (1996) proposed that intraspecific variance of mt genomes may stem from (1) rapid mt evolution, (2) sequence divergence in previously isolated populations, (3) selection acting to generate and maintain variability and (4) unusually structured or large populations. Thus, it is not unexpected that intraspecific divergence has developed in the mt genome of a globally invasive species with complex genetic population structures that are capable of reproduction by selfing such as *P. acuta* (Escobar, Nicot & David, 2008; Albrecht *et al*., 2009). The occurrence of variant mt genomes in *P. acuta* may result from putative mitochondrial introgression (Ballard & Whitlock, 2004), but more data are needed to test this hypothesis. These considerations and the findings in this study suggest that it may be informative for molecular sequence-based taxonomic identification of snails to employ combined analyses of sequences encoded by both mitochondrial and nuclear genomes.

A standard ancestral gene pattern has been postulated for molluscan mt genomes (Ki *et al*., 2010), but frequent and extensive rearrangements have led to highly diverse patterns of gene order in mt genomes across the phylogeny of molluscs (Boore *et al*., 2004; Grande *et al*., 2008). The mt genomes of Panpulmonata, however, display a relatively standard gene order with modest variations in the relative positions of tRNA genes and only rarely of protein-encoding genes (Kurabayashi & Ueshima, 2000; Knudsen *et al*., 2006; Grande *et al*., 2008). In light of the apparent standard gene pattern it was surprising that the gene order of the mt genome of *P. acuta* differed radically from that of phylogenetically close relatives within the Panpulmonata (Fig. 5). It remains unclear what mechanisms underlie the rearrangements of the mt genomes in this group (Grande *et al*., 2008; White *et al*., 2011), but the analysis of the mt gene order of *P. acuta* relative to the standard panpulmonate genome favours a combination of segmental duplication and selective deletion of supernumerary genes (Kurabayashi & Ueshima 2000; Knudsen *et al*., 2006; Grande *et al*., 2008) (Fig. 5; Supplementary Material Table S5). Despite extensive gene rearrangements, the mt genome of *P. acuta* still reflects the common mt gene order shared by many Panpulmonata. The directionality of gene homologues is the same and complements of genes that are encoded on either the H and L strands are identical. Several clusters of genes with the same relative internal positions as seen in other panpulmonates were identified in the divergent gene pattern of the mt genome of *P. acuta*. As proposed for other panpulmonates (Grande *et al*., 2008; White *et al*., 2011), the location of the POR of *P. acuta* is predicted in the intergenic region between *cox3* and *trnI* (Fig. 3), within a gene cluster that remained undisturbed during the rearrangements. This suggests that rearrangements involved groups of genes (segments of the mt genome) rather than individual genes.

Additional differences in the mt genome of *P. acuta vs* other panpulmonates are the longer branch lengths (Fig. 6), accelerated amino acid substitutions (relative rate test) and increased substitution rates (Table 2). These are indications that the mt genome of *P. acuta* is evolving faster than those of several other gastropods. Nevertheless, phylogenetic analysis performed with concatenated protein-encoding gene sequences place *P. acuta* in the clade Hygrophila (Fig. 6). This is in agreement with other phylogenetic trees based on mt and nuclear DNA sequences (Grande *et al*., 2008; Klussman-Kolb *et al*., 2008; Jörger *et al*., 2010; Dayrat *et al*., 2011; White *et al*., 2011). A number of processes may account for increased branch lengths and the increased dN/dS rates: (1) increased substitution rates that create a spectrum of mutations which may generate increased amino acid replacements, (2) relaxation of selection which could allow for an increase in the number of substitution sites, (3) mechanistic flaws in replication and/or mismatch DNA repair, (4) population effects such as bottlenecking or reproduction, especially because *P. acuta* is a simultaneous hermaphrodite that is capable of selfing (Neiman *et al*., 2010). Finally, increased substitutions rates may be explained by genome rearrangements via genome duplication and selective loss of genes.

In summary, two isolates of *P. acuta* that appear side by side in the Stubblefield Lake in New Mexico have highly similar ITS1 and ITS2 sequences, yet display high mt sequence divergence and differ considerably in length of their mt genomes. Few studies provide the entire mt genome from multiple individuals of the same species among Gastropoda, but none of these match the intraspecific sequence divergence of entire mt genome

sequences as seen within *P. acuta*. The physid snails have an mt gene order that is strikingly different from the relatively conserved pattern previously described from within panpulmonates and phylogenetic analysis indicates overall elevated substitution rates, yet phylogenetic placement of *P. acuta* remains within Hygrophila (Panpulmonata). The mt genomes from *P. acuta* may be used in future studies of topics such as intra- and interspecific sequence divergence, genome evolution and establishing phylogeny aided by gene rearrangements. We conclude that White *et al*. (2011) correctly assumed that with increased mt genomes being sequenced, there would be increased detection of gene rearrangements. Also, Boore (1999) validly cautioned against interpretation of phylogenetic relationships solely based on mt gene rearrangements within Mollusca due to the phylum's myriad of gene rearrangements, which are not connected with any type of molecular clock. It appears that *P. acuta* provides an intriguing example of the diversity of mt genomes within Mollusca.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Journal of Molluscan Studies* online.

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