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 2029, University of New Mexico, 1 University Blvd NE, Albuquerque, NM 87131, USA

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

(Received 17 October 2013; accepted 14 March 2014)

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 Cepaea nemoralis (Terrett, Miles & Thomas, 1996), Pyraminella 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 & Plenninger, 2010) and Galba peruvia (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 CO1 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

© The Author 2014. Published by Oxford University Press on behalf of The Malacological Society of London, all rights reserved

Downloaded from https://academic.oup.com/mollus/article-abstract/80/4/388/1851242 by guest on 28 July 2018
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 collars; Paraense & Pointier, 2003) were collected from Stubblefield Lake in northern New Mexico (USA) and maintained in aquaria 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).**

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

| Primer (5′–3′) | 3′ Position of primer (A/B) |
|---------------|-----------------------------|
| Pa16SF        | TAAAGTGGTATTAGATCTGACG     |
| *H3080        | AC8GATCAGTGATCCGACCAGC     |
| PaCYBF        | GGAGATCAGATCTGCGAAGACC     |
| PaCYBR        | TC8AAAGATCTGCGATATTAGCC    |
| ATP86F        | AATTCCATAAGTGCGGCTCTGAG    |
| ND3JR         | TTCTGAAGTTGCTGATCTC        |
| ATP86FC       | CCTTTGATATCCCTGATGACTGC    |
| ND4JR (B)     | ATG7CCAAGTGCAGATACCCGC     |
| *LCO1490      | GTGCACAAATCATAAGATATTGC    |
| A_CO1JRC      | AAAACTGTACGCAACATTC        |
| B_CO1JRC      | CAAAAGCAATGTCTGTAACAGC     |
| PaCO1F        | GTTTGATCGTGATATACCTGCA     |
| *LCO2198      | TA8ACTCAGAGTACCAAATG       |
| CO1JFC        | CGAGCTTATTTACACGCAACAC    |
| ND5JRC        | GAC8GTGATCTACATTCATG       |
| ND5JR         | GGA8TACCAATGTAAAGTCCAC    |
| ND5JFC        | ATCGGCTGCTAAGACGC          |
| CO2JRC        | CCTCTGAAATGTTGATGCTG       |
| ND2JRC        | ATGTCCAACTGAGTATGACC       |
| A_ND4LJFC     | TTTGGTGGCGATATGTAGTG       |
| B_ND4LJFC     | G8CCCTGAGTACCTGCTG         |
| A_ATP6JF2     | AAG8CTCAAATCTTTTGAGCAAC    |
| 12SJRC        | TGG88GCACAAATGTGAGG        |
| CO3JF         | GTTTAGGCGCAATAGCTC         |
| CO3JR         | ACCAGCTTGGATCTTACGGC       |
| CO3JFC        | CCACTGCGGATGATGAGC         |
| ND2JRC        | GAC8TTCGGTAAACAACAGG       |
| ND2JIF        | C8CTGTGTTTATCCCGAAGACG     |
| 16SJR2        | ATACTTTTTCGGCTATCCAG       |
| N2G16SJFC     | C8CTTTCAATTTTGTGATGCTG     |
| *L2510        | CG8CTGTTTATCCAAACAT        |

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).
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’TAACAAGGTTCCTCGATGGAAT3’ (Armbruster & Bernhard, 2000) and ITS2R 5’GGTTTCGACTCTTCTGAA3’ (provided by J. Nebka, modified from that published by Wade & Mordan, 2000). Terminus 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 (QiAGEN 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 sequence. 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: 164430590 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: 164430531; 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: 15717941; DeJong et al., 2004). 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. dactylomela (GenBank: NC015088; Medina et al., 2011) and A. occidentalis (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); Orschidella: 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, each one from P. acuta isolates A and B. PCR primers (Table 1) were designed and optimized using Primer3 (Rozen & Skaltsky, 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 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 rRNA genes was based on identification of anticodon 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 hp in length containing high, localized AT richness and predictive 5’ TATA sequence repeats as seen in Drosophila (Kilpert & Podosiadloowski, 2006); (2) regions with high GC skew [(G-C)/(G + C)] (Xia, 2012) using window size 2300 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). Mitochondrial genomes were depicted graphically using Artemis (Rutherford 2011). Mt genomes were depicted linearly and aligned with other panpulmonates (Grande et al., 2008; White et al., 2011) and ITS1 and ITS2 sequences from other panpulmonates (Grande et al., 2008) for other panpulmonates (Grande et al., 2008; White et al., 2011). To predict the location of the mt sequence data.

Mitochondrial gene order: P. acuta vs other panpulmonates

Starting with cox1, the order of mt genes recorded from P. acuta isolates A and B were 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 rhaphidias (Amphiboloidei; GenBank: NC016185; White et al., 2011); Ostazzila vulcani (GenBank: NC016175) and Trimusculus reticulatus (GenBank: NC016193) (both Ellobiidae; White et al., 2011); Rhiphopocaecus grundieri (Veronicellidae; GenBank: NC016183; White et al., 2011) and O. celtica (Orschidelliidae; GenBank: NC012376; Grande et al., 2008) (both Systellomatomorpha); Albinaria carralia (GenBank: NC001761; Hatzoglou, Rodakis & Lecanidou, 1995) and C. nemoralis (GenBank: NC001816; Yamaizaki et al., 1997) (both Stylomatomorphomata); 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 bathica (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
and 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: JQ90525; B: GenBank: JQ90526) 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, nadH, C, Q, atp6, E, F, rns8, M, T, cox3, I, nad2, K, V, rnl, L1, A, cytb, G, H, L2, atp8, X, nad5, 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, nadH and trnC by 2 bp, trnI 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 trnL, upstream of nad2. This is one of the largest intergenic regions, 45,488 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 14,329 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
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 atp6, cox2 and cyt 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 ≥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
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 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 \([\frac{(G-C)}{(G+C)}]\) 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.
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 χ² = 38.01, isolate B χ² = 30.82, P < 0.00001 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;...
For *P. acuta*, the levels of intraspecific divergence were different for the nuclear ITS sequences (>98% identity) as the mitochondrial 16S and COI sequences (95.25% identity).
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 (Styloematophora) 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-Beaupre et al., 2010). However, this does not apply here; P. acuta belongs to a different molluscan class and is a simultaneous hermaphrodite. Thomas 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 supernumery 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 trnL (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 places 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.

### 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 |
| nad3* | 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 atp6. Single ratios indicate differences 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.
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.

ACKNOWLEDGEMENTS

Dr Sara V. Brant (CETI, University of New Mexico UNM) provided the field collected physid snails. Dr Jeffrey C. Neckola (UNM) provided helpful discussion and contributed primers. J.R.N. was supported in part by PREP (NIH R25 GM075149). C.M.A. acknowledges support from NIH grant number P20GM103452. U.B. was supported by NSF grant number DEB-0952342. Sequencing was performed at the Molecular Biology Facility and supported in part by NIH grant number DEB-0952342 from the National Institute of General Medical Sciences.

REFERENCES

ALBRECHT, C., KROLL, O., MORENO-TERRAZAS, E. & WIEKE, T. 2009. Invasion of ancient Lake Titicaca by globally invasive *Physa acuta* (Gastropoda: Pulmonata: Hygrophila). Biological Invasions, 11: 1821–1826.

ALTSCHUL, S.F., MADDEN, T.L., SCHAFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25: 3389–3402.

ARMBRUSTER, G.F.J. & BERNHARD, D. 2000. Taxonomic significance of ribosomal ITS-1 sequence markers in self-fertilizing land snails of *Climacospira* (Stylommatophora, Cochliopidae). Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe, 76: 11–18.

ARMBRUSTER, G.F.J. & KORTE, A. 2006. Genomic nucleotide variation in the ITS1 rDNA spacer of land snails. Journal of Molluscan Studies, 72: 211–213.

BALLARD, J.W.O. & WHITLOCK, M.C. 2004. The incomplete natural history of mitochondria. Molecular Ecology, 13: 729–744.

BANDYOPADHYAY, P.K., STEVENSON, B.J., OWENBY, J.P., CADY, M.T., WATKINS, M. & OLIVERA, B.M. 2008. The mitochondrial genome of *Conus textile*, coxl-coxII intergenic sequences and conoidae evolution. Molecular Phylogenetics and Evolution, 46: 215–223.

BOORE, J.L. 1999. Animal mitochondrial genomes. Nucleic Acids Research, 27: 1767–1280.

BOORE, J.L., MEDINA, M. & ROSENBERG, L.A. 2004. Complete sequences of the highly rearranged molluscan mitochondrial genomes of the scaphopod *Graptopecten ehren* and the bivalve *Mytilus edulis*. Molecular Biology and Evolution, 21: 492–1503.

BOUSSET, L., HENRY, P.Y., SOURROUILLE, P. & JARNE, P. 2004. Population biology of the invasive freshwater snail *Physa acuta* approached through genetic markers, ecological characterization and demography. Molecular Ecology, 13: 2023–2036.

BURCH, J.B. 1989. North American freshwater snails. Malacological Publications, Hamburg, M1.

CASTRO, L.R. & COLGAN, D.J. 2010. The phylogenetic position of *Neritimorpha* based on the mitochondrial genome of *Nerita melanotus*ag (Mollusca: Gastropoda). Molecular Phylogenetics and Evolution, 57: 918–923.

CORREA, A.C., ESCOBAR, J.S., DURAND, P., RENAUD, F., DAVID, P., JARNE, P., POINTIER, J.-P. & HURTREZ-BOUSSES, S. 2010. Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fascioliasis. BMC Evolutionary Biology, 10: 881.

DAYRAT, B., CONRAD, M., BALAYAN, S., WHITE, T.R., ALBRECHT, C., GOLDING, R., GOMES, S.R., HARASEWYCH, M.G. & MARTINS, A.M. 2011. Phylogenetic relationships and evolution of pulmonate gastropods (Mollusca); new insights from increased taxon sampling. Molecular Phylogenetics and Evolution, 59: 425–437.

DE RIJK, P., WUYTS, J. & DE WAchter, R. 2003. RnaViz2: an improved representation of RNA secondary structure. Bioinformatics, 19: 299–300.

DEJONG, R.J., EMERY, A.M. & ADEMA, C.M. 2004. The mitochondrial genome of *Biomphalaria glabrata* (Gastropoda: Basommatophora), intermediate host of *Schistosoma mansoni*. Journal of Parasitology, 90: 991–996.

DEJONG, R.J., MORGAN, J.A.T., PARAENSE, W.L., POINTIER, J.-P., AMARISTA, M., AVEZI-KUMI, F.F.K., BARBIER, A., BARBOSA, C.S., BREMOND, P., CANESI, A.P., PEREIRA DE SOUZA, C., DOMINGUEZ, C., FILE, S., GUTIERREZ, A., INCANI, R.N., KAWANO, T., KAZIBWE, F., KIPIKI, J., LWAMBO, N.J.S., MIMPFOUNDI, R., NJIOKOU, F., PODA, J.N., SENE, M., VELASQUEZ, I.E., YONG, M., ADEMA, C.M., HOFKIN, B.V., MKOJII, G.M. & LOKER, E.S. 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae), with implications regarding its role as host of the human Bloodfluke, *Schistosoma mansoni*. Molecular Biology and Evolution, 18: 222–239.

DILLON, R.T., JR. & FRANKIS, R.C. 2004. High levels of mitochondrial DNA sequence divergence in isolated populations of freshwater snails of the genus *Goniobasis* Lea, 1862. American Malacological Bulletin, 19: 69–77.

DILLON, R.T., JR., WETHINGTON, A.R. & LYDEARD, C. 2011. The evolution of reproductive isolation in a simultaneous hermaphrodite, the freshwater snail *Physa*, *BMC Evolutionary Biology*, 11: 144.

DOUCET-BEAUPRE, H., BRETON, S., CHAPMAN, E.G., BLIER, P., BOGAN, A.E., STEWART, D.T. & HOEH, W.R. 2010. Mitochondrial phylogenogenetics of the Bivalvia (Mollusca): searching for the origin and mitogenomic correlates of doubly uniparental inheritance of mtDNA. BMC Evolutionary Biology, 10: 50.

DRAPARNAUD, J.P.R. 1805. *BMC* Evolutionary Biology. *Physa* In: Histoire naturelle des mollusques terrestres et fluvialitiers de la France. Montpellier, Paris.

ESCOBAR, J.S., NICOT, A. & DAVID, P. 2008. The different sources of invasion of ancient Lake Titicaca by globally invasive *Physa acuta* (Gastropoda: Pulmonata: Hygrophila). Biological Invasions, 11: 1821–1826.

FELDMANN, C.S., BREMOND, P., CANESE, A.P., PEREIRA DE SOUZA, C., DOMINGUEZ, C., FILE, S., GUTIERREZ, A., INCANI, R.N., KAWANO, T., KAZIBWE, F., KIPIKI, J., LWAMBO, N.J.S., MIMPFOUNDI, R., NJIOKOU, F., PODA, J.N., SENE, M., VELASQUEZ, I.E., YONG, M., ADEMA, C.M., HOFKIN, B.V., MKOJII, G.M. & LOKER, E.S. 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae), with implications regarding its role as host of the human Bloodfluke, *Schistosoma mansoni*. Molecular Biology and Evolution, 18: 222–239.

GRANDA, C., TEMPLADO, J. & ZARDOYA, R. 2008. Evolution of gastropod mitochondrial genome arrangements. *BMC Evolutionary Biology*, 8: 61.

HALL, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 93–96.
HATZOGLOU, E., RODAKIS, G.C. & LECANIDOU, R.L. 1995. Complete sequence and gene organization of the mitochondrial genome of the land snail Albinaria oeculea. Genetics, 140: 1353–1366.

JANNOTTI-PASSOS, L.K., RUIZ, J.C., CALDEIRA, R.L., MURTA, S.M., COELHO, P.M. & CARVALHO, O.S. 2010. Phylogenetic analysis of Biomphalaria tenagophila (Orthogly) (Mollusca: Gastropoda). Memórias do Instituto Oswaldo Cruz, 105: 504–511.

JEX, A.R., HALL, R.S., LITTLEWOOD, D.R.J. & GASSER, R.B. 2010. An integrated pipeline for next-generation sequencing and annotation of mitochondrial genomes. Nucleic Acids Research, 38: 522–533.

JÖRGER, K.M., STOGER, I., KANO, Y., FUKUDA, H., KNIELSBERGER, T. & SCHROEDER, M. 2010. On the origin of Acoclidia and other enigmatic eutheleuran gastropods, with implications for the systematics of Heterobranchia. BMC Evolutionary Biology, 13: 52.

KAYAL, E., ROURE, B., PHILIPPE, H., COLLINS, A.G. & KEFFORD, B.J. & NUGEGODA, D. 2005. No evidence for a critical transition of snails to slugs dating back to the Paleozoic, based on mitochondrial mitogenomics. BMC Evolutionary Biology, 5: 9.

KLUSSMANN-KOLB, A., DINAPOLI, A., KUHN, K., STREIT, B. & ALBRECHT, C. 2008. From sea to land and beyond – new insights into the evolution of eutheleuran Gastropoda (Mollusca: Pulmonata, Pupillidae) group of minute North American land snails. BMC Genomics, 9: 38.

KOSAKOVSKY-POND, S.L. & FROST, S.D.W. 2005. A genetic algorithm approach to detecting lineage-specific variation in selection pressure. Molecular Biology and Evolution, 22: 478–485.

KURABAYASHI, A. & UESHIMA, R. 2000. Complete sequence of the mitochondrial DNA of the primitive opisthobranch gastropod Pupa striata: systematic implication of the genome organization. Molecular Biology and Evolution, 17: 266–277.

LEE, Y.S., LEE, S.G., KANG, S.W., JEONG, J.E., BAEK, M.K., CHOI, S.H., CHAE, S.H., JO, Y.H., HAN, Y.S. & PARK, H.S. 2011. Expressed sequence tag analysis of Physa acuta: a freshwater pulmonate in Korea. Journal of Shellfish Research, 30: 127–132.

LIU, G.H., WANG, S.Y., HUANG, W.Y., ZHAO, G.H., WEI, S.J., SONG, H.Q., MU, J.Y., LIN, R.Q., ZHOU, D.H. & ZHU, X.Q. 2009. The complete mitochondrial genome of Galba persa (Gastropoda: Mollusca), an intermediate host snail of Fasciola spp. PLoS ONE, 4: e71427.

MADSEN, H. & FRANDSEN, F. 1989. The spread of freshwater snails including those of medical and veterinary importance. Acta Tropica, 46: 139–146.

MAS-COMA, F., VALERO, M.A. & BARGUES, M.D. 2009. Chapter 2. Fasciola, hymenoids and human fasciosis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in Parasitology, 69: 41–146.

MAYNARD, B.T., KERR, I.J., MCKIERNAN, J.M., JANSEN, E.S. & HANNA, P.J. 2005. Mitochondrial DNA sequence and gene organization in Australian blacklip abalone Halocetes rubra (Leach). Marine Biotechnology, 7: 645–658.

MEDINA, M., LAL, S., VALLÉS, Y., TAKAOKA, T., DAYRAT, B., BOORE, J. & GOSLINER, T. 2011. Crawling through time: transition of snails to slugs dating back to the Paleozoic, based on mitochondrial phylogenomics. Marine Genomics, 4: 51–59.

MORGAN, J.A., DEJONG, R.J., JUNG, Y., KHALLAYOUNE, K., KOCK, S., MKOJI, G.M. & LOKER, E.S. 2002. A phylogeny of planorbid snails, with implications for the evolution of Schistosoma parasites. Molecular Phylogenetics and Evolution, 25: 477–488.

NEI, M. & KUMAR, S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

NEUMAN, M., HEIMAN, G., MILLER, J.T., LOGSDON, J.M., JR. & TAYLOR, D.R. 2010. Accelerated mutation accumulation in asexual lineages of a freshwater snail. Molecular Biology and Evolution, 27: 954–963.

NEKOLA, J.C., COLES, B.F. & BERGTHORSSON, U. 2009. Evolutionary pattern and process within the Vertoq gouldii (Mollusca: Pulmonata, Pupillidae) group of minute North American land snails. Molecular Phylogenetics and Evolution, 53: 1010–1024.

PALUMBI, S., MARTIN, A., ROMANO, S., McMILLAN, W.O., STICE, L. & GRABOWSKI, G. 1991. The simple fool’s guide to PCR, version 2.0. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

PAPADOPOULOS, J.S. & AGARWALA, R. 2007. COBALT: constraint-based alignment tool for multiple protein sequences. Bioinformatics, 23: 1073–1079.

PAREANSE, W.L. & POINTIER, J.P. 2003. Physa acuta Draparnaud, 1805 (Gastropoda: Physidae): a study of totopycic specimens. Memórias do Instituto Oswaldo Cruz, 98: 513–517.

PINCIEL, J., JORDAENS, K. & BACELJAU, T. 2005. Extreme mtDNA divergences in a terrestrial snail (Gastropoda, Pulmonata, Arionidae), accelerated evolution, allopatric divergence and secondary contact. Journal of Evolutionary Biology, 18: 1264–1290.

RAWLINGS, T.A., MACINNIS, M.J., BIETER, R., BOORE, J.L. & COLLINS, T.M. 2010. Sesile snails, dynamic genomes: gene rearrangements in the mitochondrial genomes of a family of caenogastropod molluscs. BMC Genomics, 11: 440.

ROKAS, A. & HOLLAND, P.W.H. 2000. Rare genomic changes as a tool for phylogenetics. Trends in Ecology & Evolution, 15: 454–459.

ROZEN, S. & SKAESLTSKY, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics methods and protocols: methods in molecular biology (S. Krawetz & S.A. Misner, eds.), pp. 365–386. Humana Press, Totowa, NJ.

ROZENDAAL, J.A. 1997. Freshwater snails. In: Vector control – methods for use by individuals and communities (J.A. Rozendaal, ed.), pp. 337–356. WHO, Geneva, Switzerland.

RUTHERFORD, K., PARKHILL, J., CROOK, J., HORNSELL, T., RICE, P., RAJANDREAM, M.A. & BARRELL, B. 2000. Artemis: sequence visualization and annotation. Bioinformatics, 16: 944–945.

SÁNCHEZ-ARGÜELLO, P., FERNÁNDEZ, C. & TARAZONA, J.V. 2009. Assessing the effects of fluoxetine on Physa acuta (Gastropoda, Pulmonata) and Chironomus riparius (Insecta, Diteme) using a two-species water-sediment test. Science of the Total Environment, 407: 1937–1946.

SIMISON, W.B., LINDBERG, D.R. & BOORE, J.L. 2006. Rolling circle amplification of metazoan mitochondrial genomes. Molecular Phylogenetics and Evolution, 39: 562–567.

STOTHARD, J.R. & ROLLINSON, D. 1997. Partial DNA sequences from the mitochondrial cytochrome oxidase subunit I (COI) gene can differentiate the intermediate snail hosts Bulinus globosus and B. truncatus (Gastropoda: Planorbidae). Journal of Natural History, 31: 727–737.

STOTHARD, P. & WISHART, D.S. 2005. Circular genome visualization and exploration using CGView. Bioinformatics, 21: 537–539.

TAJIMA, F. 1993. Simple methods for testing molecular clock hypothesis. Genetics, 135: 599–607.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731–2739.

TERRETT, J.A., MILES, S. & THOMAS, R.H. 1996. Complete DNA sequence of the mitochondrial genome of Cepaea nemoralis (Gastropoda: Pulmonata). Journal of Molecular Evolution, 42: 160–168.

THOMAZ, D., GUILLER, A. & CLARKE, B. 1996. Extreme divergence of mitochondrial DNA within species of pulmonate land snails. Proceedings of the Royal Society of London B, 263: 363–368.
VALLES, Y. & BOORE, J.L. 2006. Lophotrochozoan mitochondrial genomes. *Integrative and Comparative Biology*, 46: 544–547.

WADE, C.M. & MORDAN, P.B. 2000. Evolution within the gastropod molluscs; using the ribosomal RNA gene-cluster as an indicator of phylogenetic relationships. *Journal of Molluscan Studies*, 66: 565–570.

WETHINGTON, A.R. & LYDEARD, C. 2007. A molecular phylogeny of Physidae (Gastropoda: Basommatophora) based on mitochondrial DNA sequences. *Journal of Molluscan Studies*, 73: 241–257.

WETHINGTON, A.R., WISE, J. & DILLON, R.T. 2009. Genetic and morphological characterization of the Physidae of South Carolina (Gastropoda: Pulmonata: Basommatophora), with description of a new species. *Nautilus*, 123: 282–292.

WHITE, T.R., CONRAD, M.M., TSENG, R., BALAYAN, S., GOLDING, R., MARTINS, A.M. & DAYRAT, B.A. 2011. Ten new complete mitochondrial genomes of pulmonates (Mollusca: Gastropoda) and their impact on phylogenetic relationships. *BMC Evolutionary Biology*, 11: 295.

WINNEPENNINCKX, B., BACKELJAU, T. & DE WACHTER, R. 1993. Extraction of high molecular weight DNA from molluscs. *Trends in Genetics*, 12: 407.

XIA, X. 2012. DNA replication and strand asymmetry in prokaryotic and mitochondrial genomes. *Current Genomics*, 13: 16–27.

YAMAZAKI, N., UESHIMA, R., TERRETT, J.A., TOKOBORI, S.I., KAI, M., SEGAWA, R., KOBAYASHI, T., NUMACHI, K.I., UEDA, T., NISHIKAWA, K., WATANABE, K. & THOMAS, R.H. 1997. Evolution of pulmonate gastropod mitochondrial genomes: comparisons of gene organizations of *Euhadra*, *Cepaea* and *Albinaria* and implication of unusual tRNA secondary structures. *Genetics*, 145: 749–758.