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INTRAGENOMIC SEQUENCE VARIATION AT THE ITS1–ITS2 REGION AND AT THE 18S AND 28S NUCLEAR RIBOSOMAL DNA GENES OF THE NEW ZEALAND MUD SNAIL, *POTAMOPYRGUS ANTIPODARUM* (HYDROBIIDAE: MOLLUSCA)

MARSHAL S. HOY[1] AND RUSTY J. RODRIGUEZ[2,3]

[1]*U.S. Geological Survey Western Fisheries Research Center, 6505 NE 65th Street, Seattle, WA 98155, USA;*
[2]*Symbiogenics, Seattle, WA 98105, USA; and*
[3]*Biology Department, University of Washington, Seattle, WA 98195, USA*

Correspondence: M. Hoy; e-mail: mhoy@usgs.gov

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ABSTRACT

Molecular genetic analysis was conducted on two populations of the invasive non-native New Zealand mud snail (*Potamopyrgus antipodarum*), one from a freshwater ecosystem in Devil's Lake (Oregon, USA) and the other from an ecosystem of higher salinity in the Columbia River estuary (Hammond Harbor, Oregon, USA). To elucidate potential genetic differences between the two populations, three segments of nuclear ribosomal DNA (rDNA), the ITS1-ITS2 regions and the 18S and 28S rDNA genes were cloned and sequenced. Variant sequences within each individual were found in all three rDNA segments. Folding models were utilized for secondary structure analysis and results indicated that there were many sequences which contained structure-altering polymorphisms, which suggests they could be nonfunctional pseudogenes. In addition, analysis of molecular variance (AMOVA) was used for hierarchical analysis of genetic variance to estimate variation within and among populations and within individuals. AMOVA revealed significant variation in the ITS region between the populations and among clones within individuals, while in the 5.8S rDNA significant variation was revealed among individuals within the two populations. High levels of intragenomic variation were found in the ITS regions, which are known to be highly variable in many organisms. More interestingly, intragenomic variation was also found in the 18S and 28S rDNA, which has rarely been observed in animals and is so far unreported in Mollusca. We postulate that in these *P. antipodarum* populations the effects of concerted evolution are diminished due to the fact that not all of the rDNA genes in their polyploid genome should be essential for sustaining cellular function. This could lead to a lessening of selection pressures, allowing mutations to accumulate in some copies, changing them into variant sequences.

INTRODUCTION

The New Zealand mudsnail (*Potamopyrgus antipodarum*), a native of New Zealand and adjacent islands (Ponder, 1988; Gangloff, 1998), has become an invasive species and spread into many aquatic ecosystems (Alonso & Castro-Díez, 2008) on four continents including North America (Alonso & Castro-Díez, 2008). It has been reported that *P. antipodarum* is found in brackish water (Costil, Dussart & Daguzan, 2001), but it is commonly described as a freshwater species (Wallace, 1992; Lively & Jokela, 2002; Dybdahl & Kane, 2005). First found in the tributaries of the Snake River (Idaho, USA) in 1985 (Bowler, 1991), it has spread into aquatic ecosystems in all western states except New Mexico (Berson, 2010). *Potamopyrgus antipodarum* has been found in diverse aquatic habitats including rivers, streams, estuaries, lakes, ponds and canals and inhabits a variety of substrates such as silt, sand, mud, cobble, riffle, run and vegetation (Crosier & Malloy, 2005). In addition to being tolerant of a wide range of salinities, *P. antipodarum* possesses additional traits that can contribute to its spread as an invasive species: (1) high fecundity (Zaranko, Farara & Thompson, 1997; Richards, 2002); (2) reproduction almost exclusively via parthenogenesis (Lively, 1992), which is an attribute shared by many successful invasive species (Lynch, 1984; Jacobsen & Forbes, 1997; Haag & Ebert, 2004); (3) low susceptibility to native predators due to its hard shell and solid operculum (Vinson & Baker, 2008); (4) ability to colonize a wide range of habitats (Alonso & Castro-Díez, 2008); (5) tolerance of a range of abiotic conditions

(Møller, Forbes & Depledge, 1994; Alonso & Camargo, 2003). Although not tolerant of temperatures below 0 °C at any salinity (Hylleberg & Siegismund, 1987), *P. antipodarum* is adept at withstanding periods of cold or desiccation by burying in the sediment (Duft *et al*., 2003).

The densities of *P. antipodarum* in rivers of the western USA can be extremely high. In the mid-Snake River, estimates of *P. antipodarum* densities range from 10,000 to 500,000/m^2 depending on habitat and season (Bowler, 1991). It is the very high reproductive rate that presents the greatest threat to native benthic organisms. As an example, within 4 years of its discovery in the Snake River, *P. antipodarum* was the dominant species in entire sections of the river (Bowler, 1991). Subsequently, five mollusc species native to the Snake River were federally listed as 'threatened' or 'endangered', in part due to the population explosion of *P. antipodarum*. As with other invasive species *P. antipodarum* may have a range of impacts on native benthic species, depending upon the site properties and the structure of the host community (Left, Burch & McArthur, 1990; Strayer, 1999). In sites with high *P. antipodarum* population densities, impacts on the carbon and nitrogen cycles within the ecosystem, and in turn the nutrient cycles, have been documented, which can have a negative impact on occupied ecosystems (Strayer, 1999; Hall, Tank & Dybdahl, 2003; Carlsson, Brönmark & Hansson, 2004).

In 1999, *P. antipodarum* was found during the benthic sampling of an Environmental Monitoring and Assessment Program (EMAP) in oceanic reaches of both the Rouge River and Columbia River estuaries (Stoddard, 2006), probably arriving there via the movement of aquaculture products or possibly carried by anglers or biologists (Zaranco, Farara & Thompson, 1997; Dybdahl & Drown, 2011). *Potamopyrgus antipodarum* has established populations in the Columbia River estuary up to its mouth at the Pacific Ocean and is exposed to fluctuating salinity, up to near full seawater levels (~34 Practical Salinity Units, PSU) (Hoy *et al*., 2012). Studies suggest that the maximum salinity tolerance of *P. antipodarum* is 26–28 PSU (Winterbourn, 1970; Costil *et al*., 2001). Therefore, the Columbia River population appears to tolerate salinity levels exceeding those previously recorded. The expansion of *P. antipodarum* into high salinity habitats demonstrates a high degree of adaptability. In North America *P. antipodarum* is predominantly represented by one clonal lineage (Dybdahl & Lively, 1995; Dybdahl & Drown, 2011) and the *P. antipodarum* that invaded estuaries in the Pacific Northwest may be of the same genotype(s) that inhabit western US freshwater lakes and streams. It is evident that these snails may have sufficient genetic or epigenetic plasticity to allow them to tolerate a large range of environmental conditions (Dybdahl & Kane, 2005). Genetic diversity is required for evolutionary adaptation, but this appears to be inconsistent with the plasticity exhibited by the clonal populations of *P. antipodarum* in the western USA. However, species with general-purpose genotypes have been associated with invasiveness (Baker, 1965) and *P. antipodarum* might be able to occupy a wide range of niches because it maintains high fitness through phenotypic plasticity (Richards *et al*., 2006; Dybdahl & Drown, 2011). It appears that *P. antipodarum* has the ability to compensate for a lack of genotypic diversity through an ability to tolerate a wide range of abiotic and ecological conditions (Alonso & Castro-Díez, 2008).

In a previous study (Hoy *et al*., 2012) we tested the salinity tolerance of two *P. antipodarum* populations from Oregon, USA. One population was from a higher salinity environment in the Columbia River estuary (Hammond Harbor) and the other from Devil's Lake, a freshwater lake near Lincoln City, Oregon. A component of that study was the analysis of the 16S mitochondrial DNA (mtDNA) and individuals of both populations were sequenced for phylogenetic analysis (16S mtDNA is well represented in the GenBank database for the genus *Potamopyrgus*). The 16S mtDNA analysis revealed no molecular variation between the Hammond Harbor and Devil's Lake populations. In this study we chose to continue to examine molecular variation between the two populations with analysis of the ITS regions of ribosomal RNA. The ITS region in particular was of interest because it is commonly used for analysis at the population and species level, because of its high degree of sequence variation (Harris & Crandall, 2000).

Eukaryotic genomes typically contain hundreds to thousands of tandemly repeated copies of the rDNA operon. It is generally accepted that the nucleotide sequence homogeneity reported among rDNA multigene families is sustained within individuals and even within species by 'concerted evolution', a phenomenon where members of a sequence family tend to evolve in a concerted fashion (Dover, 1982). In a species these repeats evolve together, maintaining high similarities among themselves, as a result of mechanisms such as unequal crossing-over and biased gene conversion. Nevertheless, variation among repeats within ITS has been documented in a range of taxa (e.g. Harris & Crandall, 2000; Gandolfi *et al*., 2001; Parkin & Butlin, 2004; Simon & Weiß, 2008; Freire *et al*., 2010) and there are a few reports of variation in 18S and 28S rRNA (Kreiger *et al*., 2006; Vierna, Gonzáles-Tizón & Martínez-Lage, 2009).

In the genetic analysis of these two *P. antipodarum* populations, it is important to identify whether the intraindividual variation found is from functional or nonfunctional copies (pseudogenes). An informative method for the identification of potential pseudogenes is the analysis of secondary structure. If the variant sequences possess mutations that disrupt the secondary structure, then they are likely to be pseudogenes. Secondary structure is essential for the function of rRNAs, as well as for the initial processing of the precursor transcript (Dixon & Hillis, 1993; Coté & Peculis, 2001; Thornhill, Lajeunesse & Santos, 2007). For the ITS regions, even small changes in the secondary structure prevent the formation of mature rRNAs (van der Sande *et al*., 1992; Beltrame, Henry & Tollervey, 1994; Peculis & Greer, 1998).

Given that *P. antipodarum* in the Columbia River estuary is exposed to higher salinity levels than has been observed elsewhere, we were interested in investigating potential molecular variation between the salt-tolerant population and a freshwater population. Ribosomal RNA genes and their spacers are widely used to document diversity, yet there have been relatively few reports of intragenomic variation in the rDNA of molluscs. Estimates of diversity or ecological distribution may be distorted by this variation, and phylogenetic analyses could be hindered. Our intent was to characterize and enumerate the level of intragenomic variation found among and between the two populations, and to include estimates of genetic variance and secondary structure analyses. Variant sequences were analysed for changes in structure and potential pseudogenes were identified.

MATERIAL AND METHODS

Potamopyrgus antipodarum is easily recognized by the following distinguishing characteristics: dark brown shell 4–6 mm in length (in US populations) with 4–6 strongly convex whorls and an operculum. There have been no reports of invasive *P. antipodarum* hybridizing with native snails in North America. Our motivation for DNA testing was not for species identification, but rather to analyse genetic diversity and search for putative population markers. In August 2007 *P. antipodarum* was collected from a freshwater habitat (Devil's Lake, Lincoln City, OR; 44°97.131′N, 124°03.794′W) and in September 2007 from an estuarine habitat (Hammond Marina, OR; 46°12.145′, 123°57.08′W). Prior to DNA extraction, the shell was removed and the body rinsed in sterile water and blotted dry. Total

genomic DNA was extracted from entire individuals using a modified cetyl trimethyl ammonium bromide (CTAB) plant genomic DNA extraction method (Ausubel *et al*., 2002): snail bodies were placed in 2-ml tubes containing a lysing matrix (lysing matrix 'A', MP Biomedicals) with 600 µl CTAB extraction buffer and 60 µl 20 mg/ml Proteinase-K and bead-beaten for 20 s at speed setting 4 in a Qbiogene FP120 bead beater (Qbiogene Inc., Carlsbad, CA, USA). Samples were vortexed for 20 s, then placed in a 55°C water bath for 1 h with periodic mixing. Sample tubes were centrifuged at 12,000 rpm for 5 min and supernatant transferred to a new microcentrifuge tube. One volume of chloroform/isoamyl alcohol (24:1) was added, samples centrifuged at 12,000 rpm for 10 min, the aqueous phase transferred to a new tube and 0.1 volume 10% CTAB/NaCl solution added and mixed. One volume of chloroform/isoamyl alcohol (24:1) was added, mixed, and centrifuged at 12,000 rpm for 5 min. The aqueous phase was transferred to a new tube, one volume CTAB precipitation solution added and mixed, and 0.6 volume isopropanol added, mixed and incubated overnight at room temperature. After another centrifugation at 12,000 rpm for 20 min, the supernatant was decanted and the pellet resuspended in 1 ml high-salt TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 1 M NaCl). The tubes were gently shaken until the pellets dissolved, 550 µl isopropanol added, mixed and DNA pelleted by centrifugation (12,000 rpm, 10 min). The supernatant was decanted and the pellet gently washed with 1 ml 70% EtOH. The DNA was air dried and resuspended in 100 µl of TE buffer.

The ITS complex was amplified using the primers: GastF (5′-TGCTTAAGTTCAGCGGGT) and GastR (5′-TAACAAGGTTTCCGTAGGTGAA) (Kane & Rollinson, 1994). A partial gene sequence of the 18S rDNA was amplified with the primers: Mollusc18sF (5′-CTGGTTGATCTCTGCCAGT) and Mollusc18sR (5′-CTGAGATCCAACTAGGACCTT) (Winnepenninckx, Backeljau & De Wachter, 1994), which amplify a 567-bp region of the 18S rDNA just upstream of ITS1 (5′ → 3′). A region of the 28S rDNA gene was amplified with the primers: P.aITS2F (5′-ACGTTCTACCCATCGCTG) and P.a28sR (5′-CAAGACGGGTCGGATGGA) (this primer set was designed from *P. antipodarum* submissions in the NCBI database accession numbers: AY014158, EF417135), which amplify a short region of the ITS2 sequence (37 bp) and 578 bp of the 28S rDNA, yielding a 615-bp product. PCRs consisting of 20 µl volumes contained *c*. 10 ng genomic DNA template, 2 µl of Bioline 10× NH_4 reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pM of each primer, 0.75 units of Bioline Biolase DNA polymerase, and overlaid with mineral oil. Thermal cycling was carried out using Applied Biosystems verity model #9902 as follows: 1 cycle at 94°C for 3 min and 35 cycles with the following regime: 95°C for 30 s, 1.0°/s ramp to 56°C, 56°C for 30 s, 1.0°/s ramp to 72°C, 72°C for 1 min followed by a final step of 72°C for 7 min.

Three individuals from each population were chosen for cloning rDNA PCR products using the OneShot TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Transformants were picked and grown in liquid LB media (with 10% glycerol). Cultures were then transferred to 96-well plates and sent to High-Throughput Sequencing Solutions (Department of Genome Science, University of Washington) for purification and sequencing.

All sequences were fully sequenced with both forward and reverse overlapping reads, and for sequence analysis were aligned into contigs and manually edited using the SEQUENCHER sequence assembly program. The sequences were compared against the GenBank database using BLAST, to verify that the sequences were from *P. antipodarum*. Unique sequences from 18S rDNA, 28S rDNA and ITS1-5.8S rDNA-ITS2 were submitted to GenBank (28S rDNA sequences accession numbers: JF960417-54; 18S rDNA: JF960455-71; ITS1-5.8S rDNA-ITS2: JF960472-509). Using Arlequin v. 3.5 (Excoffier & Lischer, 2010) a hierarchical analysis of molecular variance (AMOVA) was performed, generating estimates of genetic variation among clones within an individual, between individuals and between populations. Other descriptive measures of genetic variation among clones (intra-individual) were calculated, including mean number of sequences and mean nucleotide diversity. Nucleotide diversity (π) (Nei & Li, 1979) describes the degree of polymorphism within a population, and is defined as the average number of nucleotide differences per site between any two DNA sequences chosen randomly from the same population. Among individuals, the number of transitions, transversions and substitutions were calculated. Transitions and transversions are point mutations, with transitions being the more common exchange of a purine for a purine (A ↔ G) or a pyrimidine for a pyrimidine (C ↔ T), while transversions are an exchange of purine for a pyrimidine or a pyrimidine for a purine (C/T ↔ A/G).

For each unique sequence variant identified in this study, folding models were generated using Mfold v. 3.5 (Zucker, 2003), with all conditions and settings set to the program's default modes. Folding models for 18S and 28S rDNA partial gene sequences and the ITS2 did not include any of the flanking sequences (ITS1 for 18S rDNA, 5.8S rDNA and 28S rDNA for ITS2 and ITS2 for 28S rDNA). These putative models were evaluated against the consensus sequence structure to assess variability, which involved examining common stems, loops and bulges.

RESULTS

Direct sequencing of PCR amplicons derived from the rDNA ITS regions (ITS1-5.8S-ITS2 complex) did not yield usable sequence data due to multiple templates present in the reaction, resulting in chromatograms with mixed signal sequencing traces. Subsequent cloning of PCR amplicons of the ITS region revealed a high level of intragenomic variation. When intragenomic variation was found in the ITS, as putative controls we chose to also clone the 18S and 28S rDNA which are the genes flanking ITS. This was done to help elucidate whether the ITS in these *P. antipodarum* have inherently high variability or whether the variability could be attributable to pseudogenes. Surprisingly, intragenomic variation was also observed among cloned PCR amplicons of 18S and 28S. Intragenomic variation in ITS sequences has been reported in other taxa, including molluscs (Vierna *et al*., 2009), but not in *Potamopyrgus* species and this is the first report of intragenomic variation in the 18S and 28S rDNA genes in molluscs.

A partially contiguous section of the subunit ribosomal RNA was sequenced. This included a 567-bp region of the 18S rDNA just upstream of ITS1 (5′ → 3′), and a contiguous sequence comprised of ITS1 (481 bp), 5.8S rDNA (87 bp), ITS2 (261 bp) and a partial 28S rDNA at the 5′ end (612 bp) (the 28S rDNA sequences contained a 37-bp section of the ITS2 which was removed before analysis) (Figure 1). To establish the level of intragenomic variation in the ITS region, PCR amplicons were cloned and sequenced (sequences ranged in size from 826 to 838 bp). Direct sequencing of 18S and 28S rDNA PCR products yielded high-quality reads with no variation detected between populations. In addition to the cloning of ITS, PCR amplicons from the 18S and 28S rDNA genes were cloned as putative nonvariant controls (18S rDNA sequences ranged from 562 to 567 bp, and ITS2- 28S rDNA sequences ranged from 610 to 612 bp). Three individual snails from both populations were chosen for sequence analysis of all three rDNA regions.

Table 1 shows descriptive statistics of genetic variation for all three rDNA regions, including counts of transitions and transversions, total polymorphic sites and calculations of

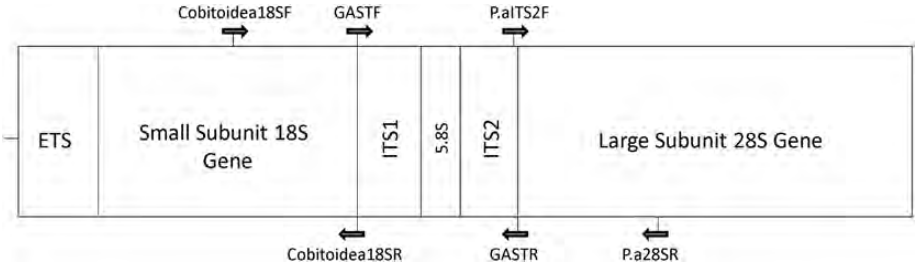

Figure 1. The rDNA gene-cluster of *Potamopyrgus antipodarum* with primer locations for PCR amplification.

Table 1. Descriptive statistics of genetic variation within individuals and among populations of *Potamopyrgus antipodarum* within the ITS, 5.8S rDNA, 18S rDNA and 28S rDNA

Gene/individual	No. of sequence variants/clones	Transitions	Transversions	Polymorphic sites	Nucleotide diversity ITS1/ITS2 (π)*
ITS1-ITS2					
Devil's Lake 1	5/6	19	6	25/754	Null/0.0047
Devil's Lake 2	7/7	17	4	21/754	0.0111/0.0038
Devil's Lake 3	8/8	22	6	27/754	0.0062/0.0043
Mean		19.33	5.33	24.33	0.0086/0.004
Hammond Harbor 1	7/7	26	6	32/754	Null/0.0076
Hammond Harbor 2	7/7	19	7	26/754	0/0.0069
Hammond Harbor 3	8/8	16	8	24/754	Null/0.0024
Mean		20.33	7	27.33	0/0.0056
5.8S rDNA					
Devil's Lake 1	1/6	0	0		
Devil's Lake 2	1/7	0	0		
Devil's Lake 3	1/8	0	0		
Mean		0	0		
Hammond Harbor 1	2/7	1	0	1/87	0.0045
Hammond Harbor 2	2/7	1	0	1/87	0
Hammond Harbor 3	1/8	0	0		0
Mean		0.67	0	0.67	0.0015
18S rDNA					
Devil's Lake 1	5/6	7	3	10/567	0.0035
Devil's Lake 2	5/7	3	3	6/567	0.0007
Devil's Lake 3	4/8	3	2	5/567	0.0041
Mean		4.33	2.67	7	0.0027
Hammond Harbor 1	6/7	7	5	12/567	0.0017
Hammond Harbor 2	4/7	4	3	7/567	0
Hammond Harbor 3	7/8	2	4	6/567	0.0038
Mean		4.33	4	8.33	0.0018
28S rDNA					
Devil's Lake 1	8/13	7	6	13/578	0.0051
Devil's Lake 2	7/11	8	3	11/578	0.0051
Devil's Lake 3	10/13	11	1	12/578	0.0041
Mean		8.67	3.33	12	0.0047
Hammond Harbor 1	6/6	9	1	10/578	0.0052
Hammond Harbor 2	13/20	17	2	10/578	0.0047
Hammond Harbor 3	10/11	10	2	12/578	0.0052
Mean		12	1.67	13.67	0.005

*Nucleotide diversity was calculated after removal of potential pseudogenes from dataset.

nucleotide diversity. Through secondary structure analyses, sequence variants that were identified as potential pseudogenes were excluded from assessments of nucleotide diversity (in ITS1 some of the individuals did not have any nonvariant sequences and were labelled 'null' in Table 1). In the cloned rDNA and ITS sequences from the three individuals from each population, three of four gene/gene regions from the Hammond Harbor population had a higher mean number of transitions (equal mean transitions for both populations in 18S rDNA), and all of the Hammond Harbor individuals

had higher mean number of total polymorphic sites and nucleotide diversity.

ITS

We sequenced 842 bp of the ITS region. Three individuals from Hammond Harbor and three individuals from Devil's Lake were sequenced, yielding 43 clones. The Devil's Lake snail clones ($N = 21$) comprised 14 unique sequences, the Hammond Harbor snail clones ($N = 22$) comprised 17 unique sequences and 11 sequences were shared between populations (given that nearly as many variant sequences were found as there were clones, this is not a definitive catalogue but rather an attempt to characterize the level of diversity found in *P. antipodarum* rDNA). The distribution of polymorphic sites was as follows: 21 sites unique to Devil's Lake, 31 sites unique to Hammond Harbor and 33 sites were shared in clones from both populations, for a total of 85 variable sites out of 842 (9.5%). These polymorphic sites were distributed throughout the ITS1 and ITS2. The 481 bp sequenced from ITS1 contained 62 polymorphic sites (12.9%), while the 262 bp sequenced from ITS2 contained 18 polymorphic sites (6.9%). Though not extensively reported, ITS intragenomic variation in molluscs appears to be highly variable between species, with the variation of polymorphic nucleotide positions in ITS1 ranging from 0.2% to 19% (Armbruster & Korte, 2006; Freire, 2010). The short 5.8S nrRNA gene (87 bp), embedded in between the ITS1 and ITS2, had three polymorphic sites (3.4%). Although not previously reported in Mollusca, the intragenomic nucleotide diversity observed in the 5.8S rDNA is similar to that reported in other taxa (Vollmer & Palumbi, 2004; Drábková *et al*., 2009).

18S rDNA

A 566-bp fragment at the 3′ end of the 18S rDNA was used for analysis. Though much lower than that observed in the ITS, the 18S rDNA also contained multiple sequences within individuals. In the Devil's Lake individuals 11 sequences were identified among 20 clones, and in Hammond Harbor individuals there were 14 sequences within the set of 23 clones. The two most frequent observed sequences (out of 23 total sequences) were found in individuals from both populations. They occurred in 40.0% and 15.0% of the Devil's Lake clones and 17.4% and 30.4% of the Hammond Harbor clones. All other sequences observed were represented by single clones. Within the 566-bp fragment of 18S rDNA, 26 sites were polymorphic (4.6%) with one 6-bp cluster of polymorphic nucleotides representing the majority (67.18%) of the variation.

28S rDNA

For 28S rDNA, a partial gene sequence spanning from the 5′ end of the gene to 578 bp downstream was used for analysis (the sequence amplified by the P.aITS2F/P.a28sR primer pair includes a 37-bp fragment of the ITS2, which was removed for the 28S rDNA analysis). Similar to the ITS and 18S rDNA sequences, multiple sequences of the 28S rDNA fragment were identified. Among the 35 cloned sequences from the Devil's Lake individuals, there were 23 unique sequences, and among the 39 clones from Hammond Harbor there were 15 unique sequences. Within the 578-bp sequence of all 74 cloned sequences, there were 59 polymorphic sites (10.2%). Supplementary Material, Figure S1 shows the positions of polymorphic bases in alignments of ITS1, 5.8S rDNA, ITS2, 18S rDNA and 28S rDNA.

Of the three rDNA genes and the ITS, significant genetic differentiation was found in the ITS and 5.8S rDNA (Table 2). The source of significant differentiation in the ITS was found between populations ($P = 0.006$) and among clones within an individual ($P = 0.042$). In the 5.8S rDNA significant differentiation was found among individuals within populations ($P = 0.003$). AMOVA of 18S and 28S rDNA did not show significant differentiation ($P = 0.658$ and $P = 0.527$, respectively). The vast majority of the variation occurred within individuals for the ITS1-ITS2, 18S rDNA and 28S rDNA, whereas minimal differences were found within populations or between the two habitats. In the ITS regions, the percentage of variation was 80.2% within individuals, and analysis of variance at the population level showed that the within-individual variation is even higher (90.13% in Devil's Lake individuals and 98.47% in Hammond Harbor individuals) relative to the variation seen among the populations. The AMOVA for the 18S rDNA also revealed that the variation was predominantly found within individuals. Testing genetic diversity between the two populations shows that nearly 99.86% of genetic variation occurred within individuals. There was a negative variance value among individuals

Table 2. Heirarchical analysis of statistically significant genetic variance using AMOVA among populations, among populations within groups (individual snails), among individuals within populations and within individual clones of *Potamopyrgus antipodarum*.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	*P*	±
Devil's Lake *vs* Hammond Harbor ITS rDNA						
Between populations	1	25.15	0.39	7.25	0.006	0.003
Among individuals within populations	4	37.517	0.33	6.13	0.196	0.012
Among clones within individuals	34	170.583	0.346	6.42	0.042	0.005
Within individual clones	40	173	4.325	80.2	0.097	0.009
Total	79	445.476	5.619			
Devil's Lake *vs* Hammond Harbor 5.8S rDNA						
Between populations	1	0.15		−0.95	1	0
Among individuals within populations	4	0.7	0.009	11.73	0.003	0.002
Among clones within individuals	34	2		−10.78	1	0
Within individual clones	40	3	0.075	100	1	0
Total	79	5.85	0.075			

Significance tested using 1023 permutations. In the 'Among populations' sources of variation: P = probability (random value ≤ observed value); in the 'Among populations within groups', 'Among individuals within populations' and 'Within populations' source of variation: P = probability (random value ≥ observed value).

Table 3. List of each sequence identified in *Potamopyrgus antipodarum* with population origin, number of polymorphisms detected, the frequency the sequence was observed among all clones, the free energy associated with the most likely model, whether the secondary structure had structure-altering polymorphisms, and GenBank accession numbers

Gene region	Sequence variant	Population	Number of polymorphisms	Frequency observed	Free energy kcal mol^{-1}	Δ Secondary structure	Accession no.
ITS1	1	DL	4	5/43	−183.21	—	JF960472
	2	DL	8	1/43	−181.32	Y	JF960473
	3	DL	6	1/43	−187.64	Y	JF960474
	4	DL	4	1/43	−176.41	Y	JF960475
	5	DL	9	1/43	−169.67	Y	JF960476
	6	DL	3	1/43	−180.41	—	JF960477
	7	DL	7	1/43	−181.08	—	JF960478
	8	DL	4	1/43	−176.11	Y	JF960479
	9	DL	7	1/43	−183.21	—	JF960480
	10	DL	10	1/43	−169.02	Y	JF960481
	11	DL	11	1/43	−170.32	Y	JF960482
	12	DL	9	3/43	−172.17	Y	JF960483-85
	13	DL	6	1/43	−179.77	Y	JF960486
	14	DL	7	1/43	−178.51	—	JF960487
	15	HH	7	1/43	−184.07	Y	JF960488
	16	HH	13	1/43	−185.21	Y	JF960489
	17	HH	9	1/43	−178.51	Y	JF960490
	18	HH	1	1/43	−185.87	Y	JF960491
	19	HH	17	1/43	−169.97	Y	JF960492
	20	HH	7	1/43	−184.65	Y	JF960493
	21	HH	11	1/43	−180.18	Y	JF960494
	22	HH	15	1/43	−169.21	Y	JF960495
	23	HH	8	1/43	−185.87	Y	JF960496
	24	HH	4	1/43	169.02	Y	JF960497
	25	HH	13	1/43	−183.21	—	JF960498
	26	HH	5	1/43	−183.45	Y	JF960499
	27	HH	4	2/43	−183.21	—	JF960500, JF960504
	28	HH	5	1/43	−176.41	Y	JF960501
	29	HH	5	1/43	−186.20	Y	JF960502
	30	HH	16	1/43	−188.70	Y	JF960503
	31	HH	6	1/43	−176.41	Y	JF960505
	32	HH	7	1/43	−175.41	Y	JF960506
	33	HH	7	1/43	−181.76	—	JF960507
	34	HH	7	1/43	−176.36	Y	JF960508
ITS2	1	DL/HH	1	13/43	−85.83	—	JF960472, JF960474, JF960477, JF960478, JF960480, JF960486, JF960492, JF960497, JF960506
	2	DL/HH	3	2/43	−74.17	Y	JF960473, JF960507
	3	DL	1	3/43	−85.83	—	JF960475, JF960479, JF960481
	4	DL	2	3/43	−85.73		JF960476, JF960482, JF960483
	5	DL	3	1/43	−85.73	—	JF960484
	6	DL	2	1/43	−86.33	—	JF960485
	7	DL	2	1/43	−85.83	—	JF960487
	8	HH	4	3/43	−85.83	—	JF960488
	9	HH	5	1/43	−74.10	Y	JF960489
	10	HH	2	1/43	−85.73	—	JF960490
	11	HH	1	1/43	−83.95	—	JF960491
	12	HH	5	1/43	−85.83	—	JF960493
	13	HH	2	2/43	−84.95	Y	JF960494, JF960505
	14	HH	3	1/43	−85.83	—	JF960495
	15	HH	1	1/43	−82.55	Y	JF960496
	16	HH	1	1/43	−85.83	—	JF960498
	17	HH	1	1/43	−71.17	Y	JF960499
	18	HH	1	3/43	−85.83	—	JF960500-02

Continued

Table 3. *Continued*

Gene region	Sequence variant	Population	Number of polymorphisms	Frequency observed	Free energy kcal mol^{-1}	Δ Secondary structure	Accession no.
	19	HH	1	1/43	−85.83	—	JF960503
	20	HH	2	1/43	−74.17	Y	JF960504
	21	HH	4	1/43	−74.17	Y	JF960508
5.8S nrDNA	1	DL/HH		40/43	−24.49	—	JF960472-79, JF960481-91, JF960493-94, JF960496-500, JF960502-08
	2	HH	1	2/43	−25.73	Y	JF960492, JF960495
	3	HH	1	1/43	−30.20	Y	JF960501
18s nrDNA	1	DL/HH		12/43	−157.26	—	JF960455
	2	DL/HH	6	10/43	−162.78	Y	JF960456
	3	DL	1	1/43	−159.78	Y	JF960457
	4	DL	5	1/43	−161.38	Y	JF960458
	5	DL	2	1/43	−160.27	—	JF960459
	6	DL	1	1/43	−152.37	Y	JF960460
	7	DL	6	1/43	−162.78	Y	JF960461
	8	DL	2	1/43	−157.57	—	JF960462
	9	DL	2	1/43	−161.18	Y	JF960463
	10	HH	3	1/43	−161.08	Y	JF960464
	11	HH	5	1/43	−163.08	Y	JF960465
	12	HH	3	1/43	−163.07	Y	JF960466
	13	HH	7	1/43	−162.53	Y	JF960467
	14	HH	3	1/43	−159.78	Y	JF960468
	15	HH	2	1/43	−161.58	Y	JF960469
	16	HH	2	1/43	−158.78	Y	JF960470
	17	HH	4	1/43	−161.08	Y	
	18	HH	1	1/43	−160.38	Y	
	19	HH	1	1/43	−159.66	—	
	20	HH	2	1/43	−154.96	—	
	21	HH	1	1/43	−158.47	—	
	22	HH	1	1/43	−156.26	—	
	23	HH	1	1/43	−158.57	Y	
28s nrDNA	1	DL/HH		14/74	−210.52	—	JF960417, JF960419
	2	DL/HH	1	10/74	−209.12	—	JF960418
	3	DL	1	10/74	−207.60	—	JF960420, JF960450
	4	DL	2	6/74	−205.92	—	JF960421
	5	DL	4	3/74	−201.96	Y	JF960422
	6	DL	1	2/74	−207.62	—	JF960423
	7	DL	6	1/74	−210.77	—	JF960424
	8	DL	3	1/74	−208.07	—	JF960425
	9	DL	1	1/74	−209.62	—	JF960426
	10	HH	1	1/74	−212.02	—	JF960427
	11	HH	2	1/74	−203.23	Y	JF960428
	12	HH	3	1/74	−205.92	—	JF960429
	13	HH	2	1/74	−212.70	Y	JF960430
	14	HH	2	1/74	−207.77	—	JF960431
	15	HH	2	1/74	−206.69	Y	JF960432
	16	HH	3	1/74	−212.82	—	JF960433
	17	HH	2	1/74	−207.02	—	JF960434
	18	HH	3	1/74	−203.39	Y	JF960435
	19	HH	2	1/74	−211.22	Y	JF960436
	20	HH	4	1/74	−209.22	—	JF960438
	21	DL	2	1/74	−206.12	Y	JF960439
	22	DL	2	1/74	−205.52	—	JF960440
	23	DL	3	1/74	−210.20	—	JF960441
	24	DL	2	1/74	−203.78	Y	JF960442
	25	DL	1	1/74	−209.12	—	JF960443

Continued

Table 3. *Continued*

Gene region	Sequence variant	Population	Number of polymorphisms	Frequency observed	Free energy kcal mol^{-1}	Δ Secondary structure	Accession no.
	26	DL	1	1/74	−211.92	Y	JF960444
	27	DL	2	1/74	−208.62	—	JF960445
	28	DL	2	1/74	−213.49	—	JF960446
	29	DL	2	1/74	−213.40	Y	JF960447
	30	DL	3	1/74	−209.42	—	JF960448
	31	DL	3	1/74	−203.52	—	JF960449
	32	DL	3	1/74	−216.52	—	JF960451
	33	DL	1	1/74	−212.86	Y	JF960452
	34		2	1/74	−208.42	—	JF960453
	35		2	1/74	−203.04	Y	JF960454

* In Δ Secondary structure column, *Y* indicates a change in secondary structure *vs* the consensus sequence and — indicates no change. DL and HH are abbreviations for the two populations: Devil's Lake and Hammond Harbor. Sequence variants 18–23 in 18S rDNA do not have accession numbers associated with them.

within populations (−9.07%), which could be biologically significant, but typically indicates an absence of genetic structure. Finally, the 28S rDNA results were very similar to those from 18S rDNA, with 99.41% of variation being found within individual clones.

rDNA secondary structure

A summary of secondary structures predicted by Mfold for all unique sequence variants is given in Table 3, which includes a list of all sequence variants, along with the number of polymorphisms, frequency observed amongst all clones, free energy (kcal mol^{-1}) and whether the sequence had a change in secondary structure. Each variant sequence for 18S rDNA, 28S rDNA and ITS had many predicted models generated by Mfold (the highly conserved 5.8S rDNA had very few). The most likely model (lowest free energy) was chosen for analysis. To evaluate changes in secondary structure and identify possible pseudogenes, predicted folding models of variant sequences were compared with the folding model of the directly sequenced PCR consensus sequence for each respective gene/spacer region. In some models polymorphisms did not change the structure, or generated very minor changes such as a single base bulge in a stem structure or addition of bases to a hairpin loop. These were not deemed to be 'Δ secondary structure' in Table 3. Many variant sequences contained polymorphisms that changed folding patterns. Predicted models for the consensus sequence of each gene/spacer region examined compared with an example of a variant sequence model with structure-changing polymorphisms are shown in Figure 2. In 18S rDNA sequences, the majority of polymorphisms are found in one 6-bp section, which is found within one helix. Figure 2 also shows the predicted secondary structure of that helix (bp 110–212); one model is the consensus sequence reference, the second is a variant sequence with polymorphisms that do not alter its structure and the third is a variant sequence with a modified folding pattern. The high number of rRNA sequence variants found within these *P. antipodarum* individuals and the variety of structural models formed suggest that the genes and spacers in this region form structures of variable stability. With the exception of 5.8S rDNA, there were many alternative structures produced, which were all very close in free energy values, therefore it is impossible to identify which might be the true form. Another method used to detect potential pseudogenes is the identification of repeat elements and/or long deletions (Vanin, 1985), neither of which were present in this set of sequences. There were no repeat elements observed and the longest deletion found was 5 bp in ITS and 1 bp in both 18S and 28S rDNA.

DISCUSSION

Our analysis of *Potamopyrgus antipodarum* did not discover any population markers in the genes and gene regions studied. However, analysis of genetic variance using AMOVA revealed significant differentiation between the two populations in ITS and 5.8S rDNA. This could be due to the greater mean number of polymorphic sites and higher nucleotide diversity values in the three genes plus ITS found among the Hammond Harbor individuals. We have not examined why the high salinity population in the Columbia River estuary had more mutations than the freshwater population, although this could present an interesting question for future studies.

In the direct sequencing of ITS PCR amplicons, mixed-signal sequencing traces were evident, but in the analysis of sequence data of 18S and 28S rDNA PCR amplicons no polymorphisms or mixed traces were found. Due to the mixed-signal sequencing traces in directly sequenced ITS PCR amplicons, we expected to find polymorphisms among cloned sequences of the ITS PCR amplicons; however, polymorphisms were also discovered in cloned sequences of 18S and 28S rDNA. Unless there are high numbers of polymorphisms among the PCR amplicons, variation can be obscured and clean consensus sequences can contain unobserved sequence variants (Linder & Banik, 2011). Although not unprecedented, intragenomic variation in 18S, 5.8S and 28S rDNA is uncommon, having been observed in a few divergent taxa (Vollmer & Palumbi, 2004; McTaggart & Crease, 2005; Nichols & Barnes, 2005; Erwin & Thacker, 2007; Ganley & Kobayashi, 2007). Vierna *et al.* (2009) studied variation of the 5S rDNA in the bivalve genus *Ensis* and observed intragenomic variation in one of the six species (*E. directus*). To our knowledge, the present study is the first to document 18S, 5.8S and 28S rDNA intragenomic gene variation in Mollusca.

To help evaluate the nature of the variation found in these *P. antipodarum*, secondary structure analysis revealed that many of the variant sequences had folding patterns that deviated from the directly sequenced PCR consensus sequences, due to structure-disrupting mutations. This could indicate that rDNA in North American populations of *P. antipodarum* contains a high quantity of nonfunctional pseudogenes. It is difficult to suggest an evolutionary explanation for such a high level of potential pseudogenes and this could provide a question for future studies.

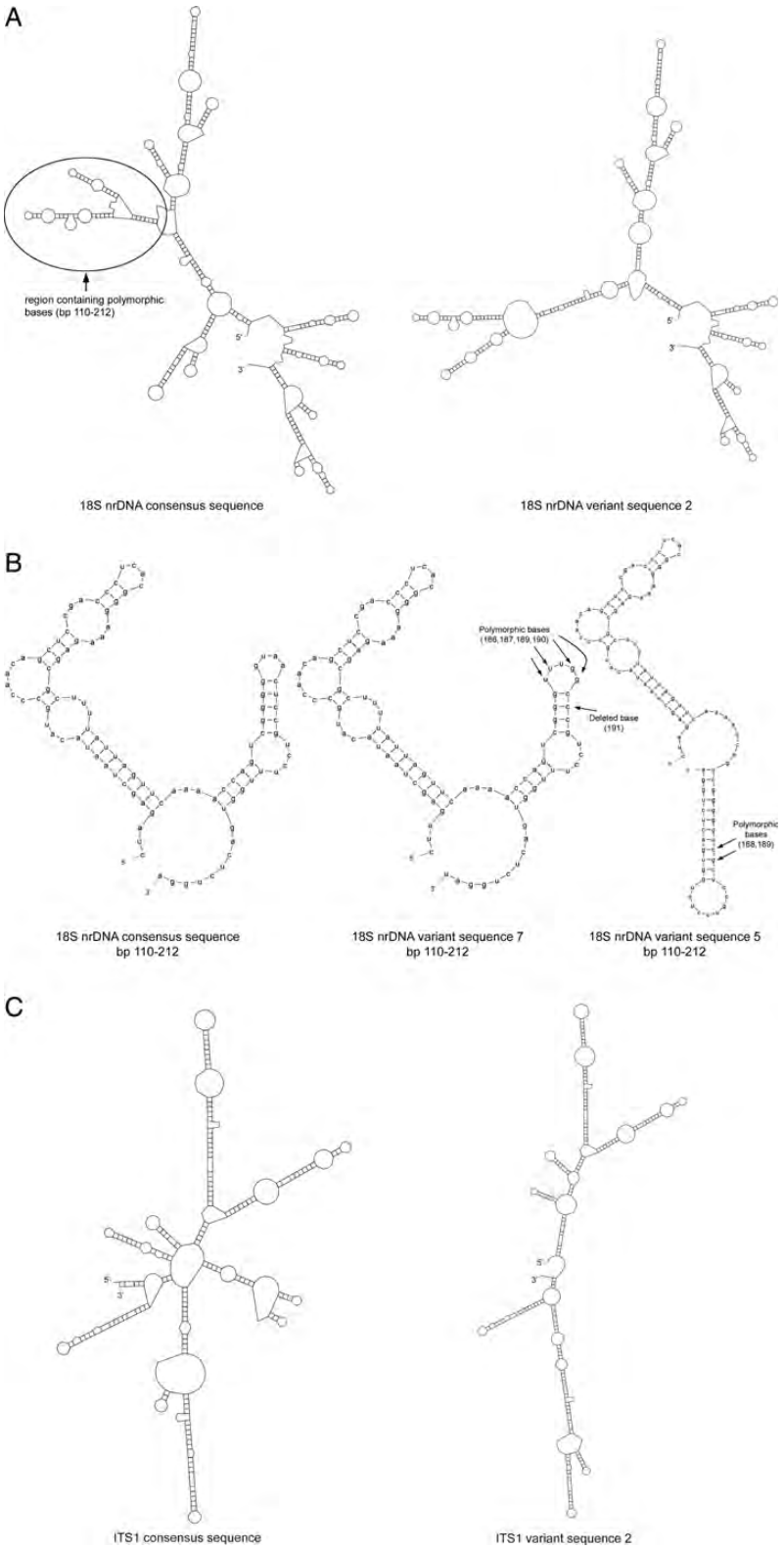

Figure 2. Secondary structure motifs for 18S rDNA, ITS1, 5.8S rDNA, ITS2 and 28S rDNA of *Potamopyrgus antipodarum*. Secondary structure of the consensus sequence folding of each gene shown together with an example of a variant sequence that contains structure-altering polymorphisms. **A.** Secondary structure of 18S rDNA consensus sequence and 18S rDNA variant sequence 2. **B.** Models of circled region (**A**) of 18S rDNA consensus sequence. Three models are shown: 18S rDNA consensus sequence (bp 110–212), 18S rDNA variant sequence 7 (bp 110–212) which contains four nonstructure-altering polymorphic bases, and 18S rDNA variant sequence 5 (bp 110–212) which contains structure altering polymorphisms. **C.** Secondary structure of ITS1 consensus sequence and ITS1 variant sequence 2. **D.** Secondary structure of 5.8S rDNA consensus sequence and 5.8S rDNA variant sequence 3. **E.** Secondary structure of ITS2 consensus sequence and ITS2 variant sequence 2. **F.** Secondary structure of 28S rDNA consensus sequence and 28S rDNA variant sequence 12.

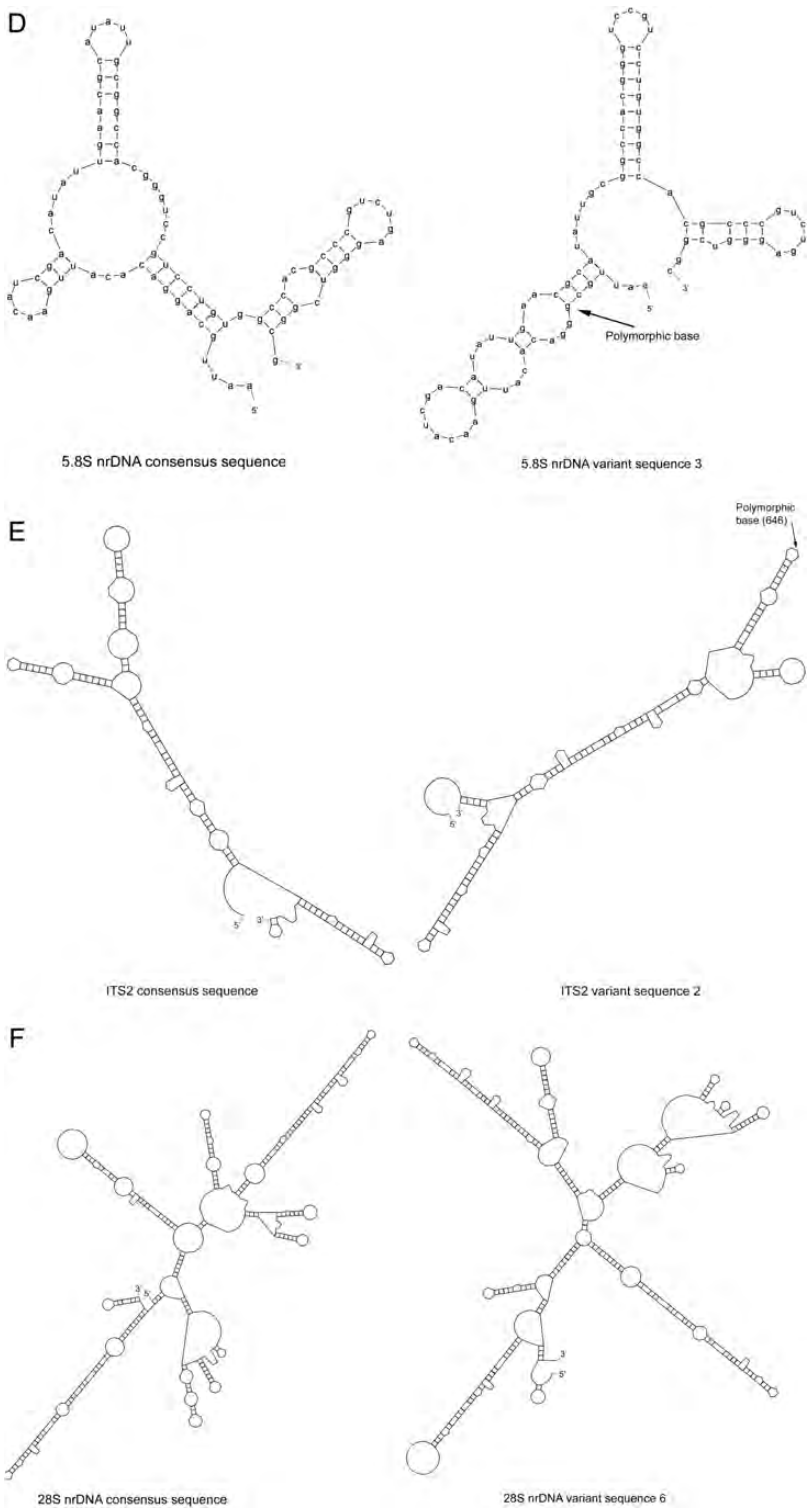

Fig. 2 *Continued*

Another avenue to explain the intragenomic variation found in *P. antipodarum* is through their polyploidy life-history. *Potamopyrgus antipodarum* is native to aquatic ecosystems throughout New Zealand, with populations exhibiting one or both of two different life-history traits (Lively, 1987; Dybdahl & Lively, 1995; Lively & Jokela, 2002): in one case dioecious with females that are sexual and diploid, while in the other the females are apomictic parthenogens producing autopolyploid progeny that are triploid clones (Phillips & Lambert, 1987; Wallace, 1992). The *P. antipodarum* collected from the two sites for our study are likely characterized by a polyploid life history, because Dybdahl & Kane (2005) stated that the introduced populations established in North America are entirely of one polyploid clonal lineage. Populations that cover extensive ranges have been shown to consist of a single clone, as determined by allozyme genetic markers. In the western USA there are six different

P. antipodarum invasion zones, but all these share an identical six-locus allozyme genotype (Dybdahl & Drown, 2011). Among the organisms in which intragenomic rDNA variability has been observed, polyploidy is a frequent attribute, although most studies have investigated this concurrence only with ITS sequences (Campbell *et al*., 1997; van Herwerden, Blair & Agatsuma, 1999; Kreiger & Fuerst, 2002; Drábková *et al*., 2009; Feher, Krak & Chrtek, 2009). In the polyploid *P. antipodarum* of North America we have demonstrated intragenomic variation not only in ITS, but in all three rDNA regions analysed.

Intragenomic variation has been found among many divergent taxa, both diploid and polyploid, although it is not a well-understood phenomenon. There are several possible explanations as to why a polyploid organism could have intragenomic variation. For example, a hybridization event could join different alleles from the two diploid parental species in allopolyploid progeny. This would produce a new genome in which intragenomic variation is present due to a number of molecular genetic processes, such as incomplete homogenization, the presence of pseudogenes or recombination (Buckler, Ippolito & Holtsford, 1997; Campbell *et al*., 1997; Hughes & Petersen, 2001). Other studies on *P. antipodarum* in North America have not reported any evidence of hybridization with native snails and the genetic data detailed in this study (and morphological data from Hoy *et al*., 2012), suggest that interspecific hybridization can be ruled out as an explanation for the presence of the observed intragenomic variation. However, it is possible that a hybridization event could have occurred before the introduction into North America. Another possible explanation for intragenomic variation in polyploid organisms is that either auto- or allopolyploidy results in an increased number of chromosomes and an increase in the number of the high-copy rDNA gene complexes. This multiplication of rDNA copies could produce an excess of copies, not all of which might be essential for sustaining cellular function. This could lead to a lessening of selection pressure, allowing mutations to accumulate in some copies and changing them into variant sequences. It is also possible that arrays of rDNA could be copied to nonhomologous chromosomes, limiting the action of concerted evolution to homogenize the multiple alleles.

The normal effects of concerted evolution appear limited and it is not known what mechanism is responsible for the lack of homogenization seen in copies of rDNA in *P. antipodarum*. Future studies involving nuclear ribosomal DNA (particularly ITS) in *P. antipodarum* should use caution because of the number of polymorphisms observed here both within and among individuals. Studies of ITS, 18S and 28S rDNA genes need to include thorough cloning to facilitate the identification of multiple sequences or paralogues within an individual and to help resolve difficulties caused by multiple sequences in phylogenetic inference. More importantly, it will be interesting to design studies to assess the relationship between organismal adaptation and intragenomic variation.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies*.

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