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AUTHORS: João P. Marques[a,*], Graciela Sotelo[a,*], Tomas Larsson[b], Kerstin Johannesson[c], Marina Panova[c,#], Rui Faria[a,d,e#]

[a]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO, Laboratório Associado, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal. joao.marques@cibio.up.pt; gsotelo.fdz@gmail.com

[b]University of Gothenburg, Department of Marine Sciences, Box 460, SE-405 30 Gothenburg, Sweden. tomas.larsson@marine.gu.se

[c]University of Gothenburg, Department of Marine Sciences, Tjärnö, SE-452 96 Strömstad, Sweden. kerstin.johannesson@marine.gu.se; marina.panova@marine.gu.se

[d]IBE, Institute of Evolutionary Biology (CSIC-UPF), Department of Experimental and Health Sciences, Pompeu Fabra University, Doctor Aiguader 88, 08003 Barcelona, Spain. rui.faria@upf.edu

[e]Animal and Plant Sciences, University of Sheffield, Shjeffield, United Kingdom. r.macieiradefaria@sheffield.ac.uk

[*]Equal contribution

[#]Corresponding authors: Rui Faria - CIBIO/InBIO Centro de Investigação em Biodiversidade e Recursos Genéticos – Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal. Phone: +351-252660406; Fax: +351-252661780; E-mail: rui.faria@upf.edu; Marina Panova - University of Gothenburg, Department of Marine Sciences, Tjärnö, SE-452 96 Strömstad, Sweden. Phone: +46-317869623; Fax: +46-317861333; E-mail: marina.panova@marine.gu.se.

Abstract

The flat periwinkles, Littorina fabalis and L. obtusata, offer an interesting system for local adaptation and ecological speciation studies. In order to provide genomic resources for these species, we sequenced their mitogenomes together with that of the rough periwinkle L. saxatilis by means of next-generation sequencing technologies. The three mitogenomes present the typical repertoire of 13 protein-coding genes, 22 transfer RNA genes, two ribosomal RNA genes and a putative control region. Although the latter could not be fully recovered in flat periwinkles using short-reads due to a highly repetitive fragment, in L. saxatilis this problem was overcome with additional long-reads and we were able to assemble the complete mitogenome. Both gene order and nucleotide composition are similar between the three species as well as compared to other Littorinimorpha. A large variance in divergence was observed across mitochondrial regions, with six- to ten-fold difference between the highest and the lowest divergence rates. Based on nucleotide changes on the whole molecule and assuming a molecular clock, L. fabalis and L. obtusata started to diverge around 0.8 Mya (0.4 - 1.1 Mya). The evolution of the mitochondrial protein-coding genes in the three Littorina species appears mainly influenced by purifying selection as revealed by phylogenetic tests based on d_N/d_S ratios that did not detect any evidence for positive selection, although some caution is required given the limited power of the dataset and the implemented approaches.

Keywords: annotation, assembly, Gastropoda, mtDNA divergence, selection

1. Introduction

Gastropods of the genus Littorina comprise interesting models for local adaptation and ecological speciation research (Johannesson, 2003), as illustrated by the many studies on ecotype evolution in the rough periwinkle L. saxatilis (Olivi, 1792) (e.g. Butlin et al., 2014; Johannesson et al., 2010; Rolán-Alvarez et al., 2004). Similarly, the flat periwinkles L. obtusata (Linnaeus, 1758) and L. fabalis (Turton, 1825), two sister species that started to diverge around 1 million years ago (Mya) (Tatarenkov, 1995), present important ecological differences that presumably have played a key role in their diversification (Reid, 1996; Williams, 1990). Notably, shared mitochondrial haplotypes suggest that mitochondrial DNA (mtDNA) introgression has occurred between the two species (Kemppainen et al., 2009). However, with the exception of one marginal population, contemporary hybridization has not been supported by nuclear markers (Carvalho et al., 2016; Kemppainen et al., 2009 and references therein). Because mtDNA introgression is known to distort phylogenetic relationships between taxa (e.g. Melo-Ferreira et al., 2012) and introgressed mtDNA haplotypes can be a source of material for adaptation in the receiver species (Llopart et al., 2014), identifying the causes of the different patterns observed with mtDNA and nuclear markers is fundamental to understand the evolutionary history of flat periwinkles.

The fact that the mitochondrial genome (mitogenome) is haploid, together with its large copy-number in the cell, a high mutation rate (relative to the nuclear genome), and absence of (or reduced) recombination, contribute to make mtDNA the marker of choice in phylogenetic and phylogeographic analyses (see Ballard and Whitlock, 2004 and references therein). Nonetheless, not all mtDNA regions are equally informative as substitution rates vary enormously across the mitogenome (e.g. Castellana et al., 2011; Simon et al., 1994). Classifying mtDNA regions according to those rates (i.e. from most conserved to hypervariable) can thus help a more informed selection of suitable mtDNA markers to address phylogenetic questions at different depths in a specific taxonomic group.

Many phylogenetic and phylogeographic studies assume that mtDNA variation is essentially neutral. However, there is evidence showing that some mtDNA mutations can be adaptive (e.g. Castellana et

al., 2011; Jacobsen et al., 2016 and references therein), and this can mislead mtDNA-based inferences on populations' demography and history (Bazin et al., 2006). Therefore, it is important to assess if mtDNA evolution in a given group is neutral or has been shaped by selection. Although disentangling these hypotheses remains a difficult task, advances in sequencing technologies now allow the analysis of complete mitogenomes in a more cost-effective manner and thus the identification of the genetic differences between species across the entire molecule, including putative adaptive mutations. Capitalizing on recent efforts to increase the genomic resources in these species, here we sequenced the mitogenomes of L. fabalis and L. obtusata, together with L. saxatilis (outgroup), with the goals of: i) characterizing their structure and composition, ii) estimating species divergence across different genes, and iii) detecting positive selection based on patterns of codon evolution. This comparative analysis of the three species provides useful information to guide the choice of mtDNA markers for further phylogenetic and phylogeographic studies in Littorina.

2. Materials and methods

2.1 Sample collection, laboratorial procedures and sequencing

Littorina fabalis (of the ME ecotype sensu Carvalho et al., 2016) and L. obtusata were collected from two distinct localities (Póvoa de Varzim and Rio de Moinhos, respectively) in Portugal in November 2012 (Table 1). Snails were taken alive to the laboratory and processed as in Carvalho et al. (2016) before molecular analysis. Briefly, genomic DNA was extracted from head-foot tissue using the CTAB method as described in Galindo et al. (2009). DNA quality was assessed by agarose gel electrophoresis and quantity was measured with Qubit using the dsDNA BR Assay Kit (Life Technologies). One adult male of each species was then selected for whole-genome sequencing. The two individuals have been genotyped for microsatellites by Carvalho et al. (2016) and represent genetically pure L. fabalis (Portuguese cluster) and L. obtusata. Library building for Illumina sequencing was carried out at CIBIO-InBIO, University of Porto (Portugal). Each sample (2 µg of DNA) was subject to four cycles of fragmentation (15 secs/90 secs - ON/OFF) on mode High (H) using a Bioruptor XL (Diagenode). Libraries (with individual barcodes for species) were constructed

with the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina) aiming at insert size of 350bp. Each library was sequenced in three lanes of a HiSeq1500 platform at CIBIO-InBIO in paired-end mode (2x100bp).

Littorina saxatilis (of the Crab ecotype sensu Johannesson et al., 2010) was collected from Saltö in Sweden in December 2010, and a single adult male was chosen for whole-genome sequencing (Table 1). DNA was extracted from fresh tissues (head-foot) using a specifically modified CTAB protocol (Panova et al., 2016). DNA quality and quantity were accessed by agarose gel electrophoresis and Nanodrop spectrophotometer. The L. saxatilis de novo genome sequencing was conducted as a part of the IMAGO Marine Genome projects of the Centre for Marine Evolutionary Biology (CeMEB), University of Gothenburg (Sweden), using both short-read (Illumina) and long-read (Pacific Biosciences - PacBio) technologies (see http://cemeb.science.gu.se/research/target-species-imago+/littorina-saxatilis for details). Library construction and sequencing were performed by Science for Life Laboratory (Sweden).

Because it was not possible to recover the complete mitochondrial sequence for flat periwinkles using Illumina reads (see section 3.1), attempts to fill this gap and close the mitogenome were performed with Sanger sequencing. Based on the complete mitogenome of L. saxatilis, and the almost complete assemblies of the two flat periwinkle species, primers were designed on flanking genes (tRNA-Phe and COX3) as well as within the non-repetitive part of the largest non-coding region, likely corresponding to the control region (CR) (primer sequences are available upon request). Successful amplification was obtained with PrimeSTAR GXL DNA polymerase (TaKaRa) in 50 µL reactions containing 1 µL of template DNA (approx. 10 ng), 10 µL of 5x reaction buffer, 4 µL of 10 mM dNTPs (2.5 mM each), 1 µL of 10 µM forward and reverse primers and 1 µL of 1.25 U/µL polymerase. PCR cycling conditions consisted of 35 cycles of denaturation at 98° for 10 s, annealing at 55° for 15 s and extension at 68° for 10 s. PCR products were visualized in 2% agarose gels and purified with Exo I and FastAP (Thermo Scientific). Sanger sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands), using the corresponding forward and reverse primers.

2.2 Assembly, validation and annotation

The partial mitochondrial sequence of L. saxatilis (GenBank accession number – a.n. AJ132137.1) was used as query in a BLASTN (Altschul et al., 1997) search using default settings against a preliminary L. saxatilis genome assembly constructed from several Illumina libraries with insert sizes ranging from 150bp to 6kb (http://cemeb.science.gu.se/research/target-species-imago+/littorina-saxatilis) with SOAPdenovo2 vr240 (Luo et al., 2012). Before assembly, reads were trimmed for quality (q>20) and length (n>35) using trim_galore v0.3.7 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), and adaptors were removed using Cutadapt (Martin, 2011) as run from within trim_galore. Contig sequences with similarity to the partial mitochondrial sequence were extracted and re-scaffolded with SOAPdenovo2 vr240, after processing through the SOAPdenovo2 v2.0 prepare module (https://sourceforge.net/projects/soapdenovo2/files/Prepare/). In order to further extend this (incomplete) assembly, PacBio read data were incorporated using the software PBJelly from PBSuite v14.7.14 (English et al., 2012). The resulting scaffold was manually curated: circularity was confirmed, redundant extremities were removed and nucleotide discrepancies associated with the incorporation of PacBio reads were corrected based on Illumina contig sequences (supported by higher coverage and lower error rate than PacBio). This final "consensus" contig was then visually inspected by re-mapping Illumina and PacBio reads with Bowtie2 v2.2.1 (Langmead et al., 2009) and BLASTN using default settings.

The inferred de novo L. saxatilis mitogenome sequence was then used as reference to map L. obtusata and L. fabalis reads with Bowtie2 v2.2.6; once raw reads were clipped to remove adaptors using Perl scripts based on Cutadapt and trimmed for quality (q>30) and length (n>50) using the script TrimmingReads.pl from the NGS QC Toolkit (Patel and Jain, 2012). For each species, mapped reads were retrieved and assembled with SPAdes v3.6.2 (Bankevich et al., 2012). This rendered an almost complete mitochondrial sequence for each species, with a long repetitive part preventing the full recovery of the putative CR (see section 3.1).

The three de novo assemblies, independently implemented for each species, were then partially validated by re-sequencing a total 12%-21% of the mitogenome using Sanger (partial putative CR and partial COX1 and CYTB for L. saxatilis; and the same plus partial ND5 for flat periwinkles - primers and conditions available upon request). Finally, the synteny revealed by the alignment of the mitogenomes of L. saxatilis, L. obtusata and L. fabalis subsequently performed (see below) further reassured the accuracy of the resulting sequences.

The three mitogenomes were annotated using MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013) to identify protein-coding (PCGs), ribosomal RNA (rRNAs) and transfer RNA (tRNAs) genes. The tRNAs were also annotated with ARWEN v1.2 (Laslett and Canbäck, 2008) and tRNAscan-SE v1.21 (Lowe and Eddy, 1997) and manually curated when inconsistencies were detected between tools. Gene limits were refined by comparison with orthologous mtDNA sequences of other Littorinimorpha (Cunha et al., 2009) and using BLASTX (Altschul et al., 1997) against the non-redundant protein sequences database in GenBank. Repeat identification was done with RepeatMasker Web Server (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). Final quality control of the annotation was performed following the recommendations in Cameron (2014). Graphical representation of L. saxatilis mitogenome (Figure 1) was drawn with OGDRAW (Lohse et al., 2007).

2.3 Sequence analyses

An initial alignment of the three mitogenome sequences was obtained with ClustalW (Thompson et al., 1994) as implemented in BioEdit v7.2.3 (Hall, 1999) and visually confirmed (Supplementary Material Online). Sequence composition and divergence (p-distance) were estimated using MEGA6 (Tamura et al., 2013). The ratio of nonsynonymous (d_N) to synonymous (d_S) substitution rates, represented as ω, was used to detect signatures of positive selection (usually inferred when $\omega > 1$) on the evolution of the PCGs in each of the three Littorina lineages. To do so, we used the branch models implemented in codeml in the PAMLX v1.3.1 package (Xu and Yang, 2013), which allow ω to vary

among branches in the phylogeny (Yang, 1998; Yang and Nielsen, 1998). By means of Likelihood ratio tests (LRT), the null model of a single ω was evaluated against: i) the free-ratios model where an independent ω is assumed per branch; and ii) the two-ratios model where a foreground branch (one at a time, three tests in total) is defined to accommodate a different ω respect to the rest (background branches). These analyses were performed gene by gene and also for the concatenated dataset (the 13 PCGs altogether).

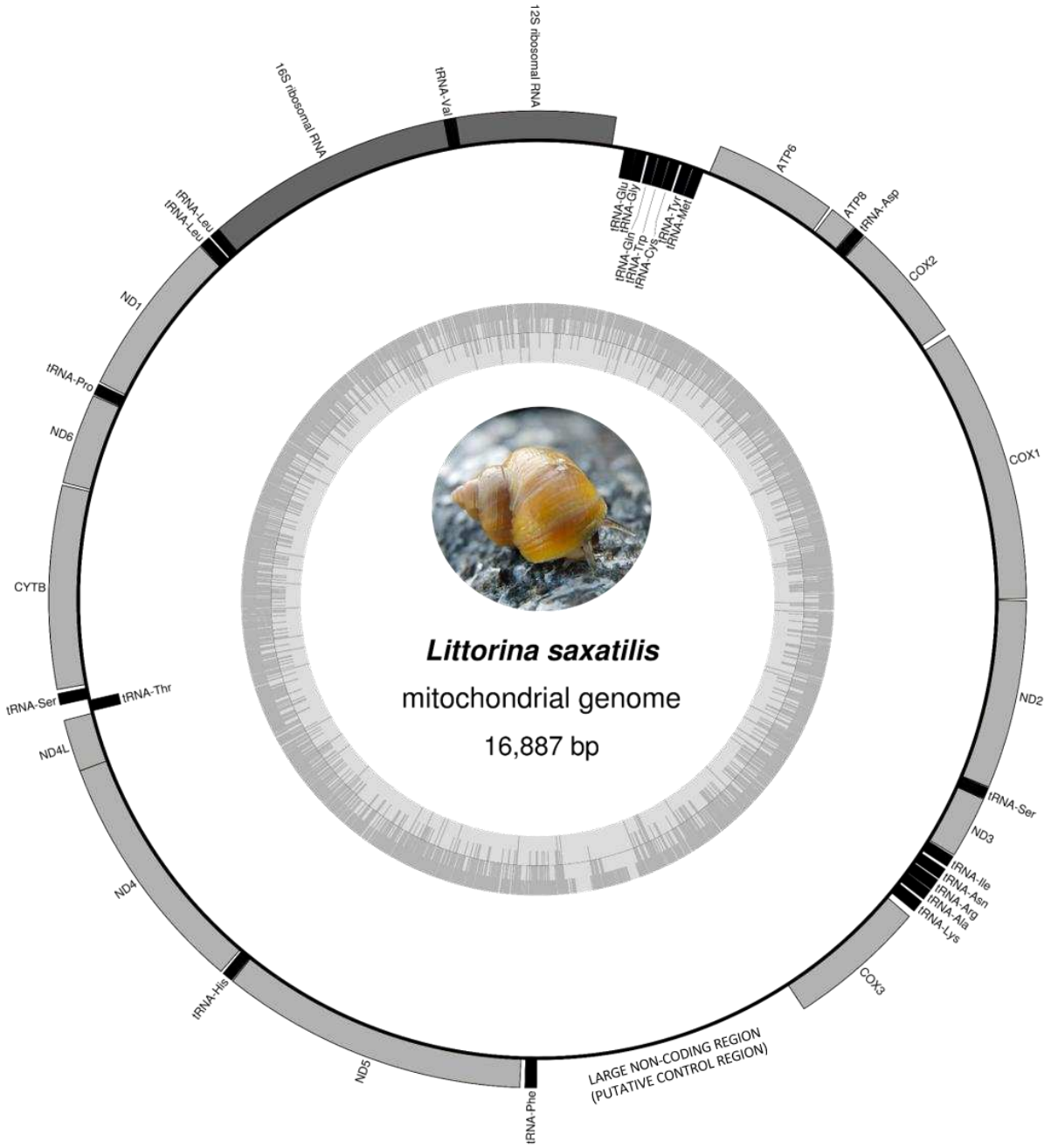
3. Results and Discussion

3.1 Mitogenome organization and composition

The complete mitogenome of L. saxatilis (GenBank a.n. KU952094; 16,887bp) and the near complete mitogenomes of L. obtusata (GenBank a.n. KU952093; 16,300bp) and L. fabalis (GenBank a.n. KU952092; 16,318bp), all supported by a coverage > 100X and partially (12%-21%) confirmed by Sanger (re-)sequencing, present the typical repertoire of 13 PCGs, 22 tRNAs, two rRNAs, and a putative CR (Table 2). The repetitive content of this region (see below) did not allow its full recovery for the flat periwinkles despite our additional efforts with Sanger sequencing. In contrast, the long-reads obtained with PacBio for L. saxatilis did span through that complex region (although it might not be completely error-free as it could not be confirmed with Sanger sequencing). This could question to what extent publicly available mitogenome sequences that have been reconstructed from short-reads could in fact be incomplete; and suggests that long-read sequencing followed by curation and validation procedures may be an efficient solution for filling gaps in repetitive regions. The PCGs encompass 11,250bp, all starting with an ATG codon and ending with a TAA or TAG codon. As an exception, the stop codon for ND4 differs between L. saxatilis (TAG) and L. obtusata - L. fabalis (TAA) (Table 2). All tRNAs were successfully annotated, forming the typical cloverleaf structure and ranging from 58 to 73bp in length. The rRNAs, 12S and 16S, are flanked by tRNA-Glu and tRNA-Leu2 and separated by tRNA-Val. All mitochondrial genes are encoded on the heavy (H) strand with the exception of eight tRNAs (Table 2, Figure 1), and gene order is the same for the three Littorina species as well as other Littorinimorpha mitogenomes except those of the superfamily Vermetoidea

(Cunha et al., 2009; Osca et al., 2015; Rawlings et al., 2010). The putative CR, located between tRNA-Phe and COX3 in the three species (Figure 1), shows two distinct parts in terms of sequence similarity between species: a relatively conserved sequence on its 5' and 3' extremes (28bp and 561bp, respectively) and a highly repetitive stretch in the middle (960bp in L. saxatilis, and at least 380bp in L. obtusata and 398bp in L. fabalis), consisting of several motifs in tandem that vary among species. The mitogenome nucleotide composition is similar between the three species, with an average of 30.1% A, 36.4% T, 19.0% C and 14.6% G, and a GC content ranging from 33.1% to 33.9% (Supplementary Table 1), and closer to those of the genera Oncomelania, Potamopyrgus and Strombus than to the remaining Littorinimorpha for which the mitogenome sequence is available (see Supplementary Table 2). The whole CR presents a higher AT content than the rest of the mitogenome (75.1% and 66.1%, respectively), as expected for this region (Lunt et al., 1998; Zhang and Hewitt, 1997).

Figure 1. Circular map of the L. saxatilis mitogenome (gene codes according to Table 2). The 13 protein-coding genes (PCGs) are represented in light grey; the 2 ribosomal RNAs (rRNAs), in dark grey; and the 13 transfer RNAs (tRNAs), in black. Genes encoded in the H strand (i.e. counterclockwise transcribed) are indicated outside the main circle, while genes encoded in the L strand (i.e. clockwise transcribed) are indicated inside. The inner circle plot represents GC content (dark grey).



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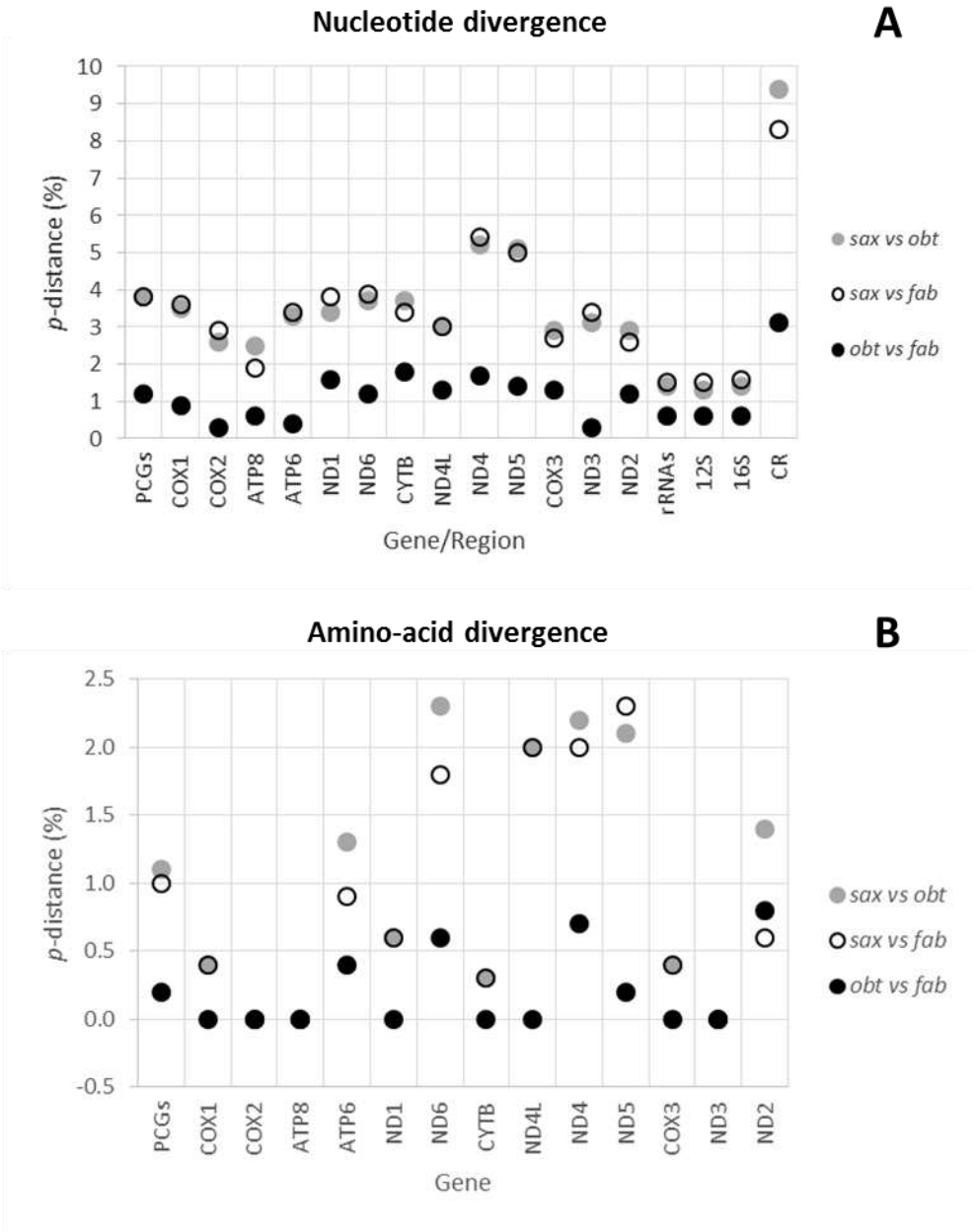
249 3.2 Divergence rates and selection

250 In agreement with their phylogenetic relationship, lower mitogenome sequence divergence is observed
251 between flat periwinkle species than between any of them and L. saxatilis. Excluding the CR, overall
252 nucleotide divergence is 1.0% between flat periwinkles and 3.3% between each of them and L.
253 saxatilis. For PCGs, nucleotide divergence ranges from 0.3% (ND3 and COX2) to 1.8% (CYTB)

between flat periwinkles, and from 1.9% (ATP8) to 5.4% (ND4) between these and L. saxatilis. For rRNAs, nucleotide divergence is 0.6% between flat periwinkles and 1.5% (average) when compared to L. saxatilis; in contrast with 3.1% and 8.9% (mean), respectively, for the CR (excluding the repetitive and non-conserved part) (Figure 2). This variation in divergence across mtDNA genes/regions (with rRNAs and CR among the most conserved and variable, respectively) conforms to the general trend described for both invertebrates and vertebrates (Simon et al., 1994). In particular, the ratio of the highest to the lowest divergence in PCGs between flat periwinkles (6.0) and between these and L. saxatilis (2.5) is within the range (1.4 to 10.1) observed for other congeneric Littorinimorpha species for which the mitogenome sequence is available (data not shown). This variation allows making a more adequate choice of markers for future phylogenetic and phylogeographic analysis in Littorina, depending on the time-scale of the questions or taxa under study. In this respect, ND4 (for coding) and CR (for non-coding) seem to be among the fastest evolving genes/regions and thus useful for addressing recent evolutionary questions. In contrast, ATP8 (and the rRNAs) are among the slowest and consequently more suitable for assessing older evolutionary events.

In terms of amino-acids, the total number of differences between species is quite low: 9 between flat periwinkles and 40 (mean) between them and L. saxatilis. This pattern of higher amino-acid divergence (p-distance) between L. saxatilis and any of the flat periwinkles than between L. fabalis and L. obtusata is observed across all PCGs except ND2 (Figure 2). Divergence between L. fabalis and L. saxatilis has been dated around 2.5 Mya (95% highest posterior density-HPD: 1.4 - 3.5 Mya), based on partial CYTB sequences and fossil calibrations (Panova et al., 2011), and 2.83 Mya based on partial 12S and 16S rRNAs and complete CYTB sequences together with fossil and geological information (Reid et al., 1996). Assuming a molecular clock, this would render a divergence time between L. fabalis and L. obtusata of about 0.8 Mya (0.4 - 1.1 Mya) according to differences along the whole molecule (excluding the repetitive part of the CR), which is in the lower range of previous estimates derived from allozymes (1.25 ± 0.47 Mya; Kemppainen et al., 2009 following Tatarenkov, 1995) or mtDNA (1.32 Mya; Reid et al., 1996).

Figure 2. Pairwise divergence across mitochondrial genes/regions among three periwinkle species: L. saxatilis (sax), L. obtusata (obt), and L. fabalis (fab). A) Nucleotide divergence. Mean values for protein-coding genes (PCGs) and ribosomal RNAs (rRNAs) are represented. Estimates for control region (CR) refer to its non-repetitive part (589bp, see section 3.1). B) Amino-acid divergence.



No signatures of positive selection (ω >1) were detected on the mitogenome of these three Littorina species. Although the null model of a single ω for all branches was rejected in two cases: the concatenated dataset with L. fabalis as foreground lineage (χ^2 = 4.206, df = 1, P < 0.05) showing lower divergence than the other lineages (ω_1 = 0.010 vs. ω_0 = 0.035, respectively), and the ND2 gene with L. obtusata as foreground lineage (χ^2 = 4.261, df = 1, P < 0.05) showing higher divergence then the other lineages (ω_1 = 0.195 vs. ω_0 = 0.023, respectively), the ω values per branch (species) were always < 1. Higher ω values (still < 1) for ND2 have been reported in several organisms, suggesting relaxed purifying selection on this gene (Jacobsen et al., 2016; Sun et al., 2011). Given that the mitogenome contains the code to synthetize proteins that, among other functions, play an essential role in the cell energy production, pervasive purifying selection as observed here is expected.

Nonetheless, signatures of positive selection in mitochondrial genes have been found in some marine animals (e.g. Foote et al., 2011; Longo et al., 2016), in some cases related with distinct metabolic demands at different temperatures. At a macrogeographic scale, flat periwinkles present a largely overlapping distribution across the European coast (from Norway to Portugal), therefore experiencing a similar thermal regime. However, at a local scale, in tidal regions of Europe L. fabalis tends to occupy the lower part of the intertidal, remaining submerged most of the time, whereas L. obtusata is more common in the mid to upper part of the intertidal, spending larger periods outside the water. Although this could impose divergent selective pressures associated with metabolism between the two species, here we did not find molecular signatures of such process. Still, the observed lack of evidence for positive selection should be taken with caution. The limited number of taxa and the relatively low divergence between species can result in low power of phylogenetic-based tests for selection (e.g. Yang, 2002). As well, adaptation could have occurred during a short period of time in a single site instead of involving multiple amino-acid substitutions on multiple sites through time (Hughes, 2007); and thus the footprints of positive selection could have been masked by purifying selection (Zang et al., 2005), making its detection difficult (Hughes, 2007; Nozawa et al., 2009). Finally, putative haplotype(s) under selection could be circumscribed to particular geographic location(s) not represented in our samples.

Alternatively, positive selection could have influenced other parts of the mitogenome not tested with this approach (focused on protein-coding genes). Namely, regions with potential regulatory functions in the CR have been suggested as the target of selection in the mitogenome, and inclusively in speciation (Burton and Barreto, 2012; Melo-Ferreira et al., 2014; Rollins et al., 2016). In particular, long arrays of repeats in the CR, as those observed here, have been implicated in the regulation of replication and transcription of the mitogenome (Hauth et al., 2005; Hirayama et al., 2010; Lunt et al., 1998; Rand, 1993). Remarkably, although the CR for flat periwinkles is not complete, partial sequences from several individuals from each species show that the repetitive motifs may differ both between and within species (data not shown), suggesting rapid evolution of this part of the mitogenome. Whether the repeats have a functional role in these Littorina taxa and are under selection, as shown for other organisms (Hirayama et al., 2010), needs to be addressed in future studies.

4. Conclusion

A remarkable variance in divergence across the mitogenome was observed for these three Littorina species, with an almost ten-fold difference between the lowest and highest rates among flat periwinkles (from 0.3% - ND3 and COX2, to 3.1% - CR) and over six-fold among flat periwinkles and L. saxatilis (from 1.4% - 12S rRNA, to 8.9% - CR). Phylogenetic tests based on d_N/d_S ratios failed to show evidence for positive selection in mitochondrial protein-coding genes, suggesting that evolution of these genes is mainly influenced by purifying selection. Although caution is necessary in the interpretation of these results, until evidence for adaptive mitochondrial evolution is collected, neutral processes driven by demographic factors (e.g. episodes where females of the rarer species mate more frequently with males from the more abundant species than in the other direction) and subsequent long-term backcrossing into one of the parental species are strong hypotheses to explain mtDNA introgression in flat periwinkles.

Nucleotide sequence accession number

The project data is available at GenBank under the a.n. PRJNA314740. The sequence associated data are MIxS compliant.

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Tables

Table 1. Mitogenome and environmental features.

Item	Description		
Organism	Littorina saxatilis	Littorina obtusata	Littorina fabalis
MIGS data			
Investigation_type	Organelle	Organelle	Organelle
Project_name	gO10	oM33	fPOV34
Collection_date	2010-12	2012-11	2012-11
Lat_lon	58.8697 N 11.1197 E	41.5667 N 8.7972 W	41.3881 N 8.7731 W
Country	Sweden	Portugal	Portugal
Environment	Marine: intertidal zone	Marine: intertidal zone	Marine: intertidal zone
Isol_growth_condt	Not applicable	Not applicable	Not applicable
Sex	Male	Male	Male
Dev_stage	Adult	Adult	Adult
Tissue	Head-foot	Head-foot	Head-foot
Sequencing_meth	Sequencing by synthesis	Sequencing by synthesis	Sequencing by synthesis
Assembly	SOAPdenovo2 vr240 + PBSuite v14.7.14	SPAdes v3.6.2	SPAdes v3.6.2
Annot_source	BLAST + MITOS	BLAST + MITOS	BLAST + MITOS
Estimated_size	16.887		
Biome	ENVO:00000569	ENVO:00000569	ENVO:00000569
Feature	ENVO:00000316	ENVO:00000316	ENVO:00000316
Material	ENVO:00002006	ENVO:00002006	ENVO:00002006
Geo_loc_name	Sweden: Saltö	Portugal: Rio de Moinhos	Portugal: Póvoa de Varzim
Genome assembly data			
Assembly method	SOAPdenovo2 vr240 + PBSuite v14.7.14	SPAdes v3.6.2	SPAdes v3.6.2
Assembly name	gO10	oM33	fPOV34
Genome coverage	> 100X	> 100X	> 100X
Sequencing technology	Illumina HiSeq + PacBio	Illumina HiSeq	Illumina HiSeq

Table 2. Mitochondrial genome annotation for L. saxatilis, L. obtusata and L. fabalis, including strand, length and location of each gene/region. Start and stop codons for each protein-coding gene are also indicated.

					L. saxatilis		L. obtusata		L. fabalis	
Gene/Region	**Strand**	**Length (bp)**	**Start**	**Stop**	**Location**	**Intergenic nucleotides[1]**	**Location**	**Intergenic nucleotides[1]**	**Location**	**Intergenic nucleotides[1]**
COX1	H	1536	ATG	TAA	1-1536	30	1-1536	30	1-1536	30
COX2	H	687	ATG	TAA	1567-2253	2	1567-2253	2	1567-2253	2
tRNA-Asp	H	69			2256-2324	1	2256-2324	1	2256-2324	1
ATP8	H	159	ATG	TAG	2326-2484	13	2326-2484	13	2326-2484	13
ATP6	H	696	ATG	TAG	2498-3193	31	2498-3193	31	2498-3193	31
tRNA-Met	L	68			3225-3292	1	3225-3292	1	3225-3292	1
tRNA-Tyr	L	68			3294-3361	11	3294-3361	11	3294-3361	11
tRNA-Cys	L	65			3373-3437	1	3373-3437	1	3373-3437	1
tRNA-Trp	L	66			3439-3504	1	3439-3504	1	3439-3504	1
tRNA-Gln	L	58			3506-3563	11	3506-3563	11	3506-3563	11
tRNA-Gly	L	67			3575-3641	-1	3575-3641	-1	3575-3641	-1
tRNA-Glu	L	71			3641-3711	72	3641-3711	72	3641-3711	72
12S rRNA	H	895/894[2]			3784-4678	-3	3784-4677	-3	3784-4677	-3
tRNA-Val	H	68			4676-4743	-22	4675-4742	-22	4675-4742	-22
16S rRNA	H	1415			4722-6136	-10	4721-6135	-10	4721-6135	-10
tRNA-Leu2	H	67			6127-6193	8	6126-6192	8	6126-6192	8
tRNA-Leu1	H	67			6202-6268	0	6201-6267	0	6201-6267	0
ND1	H	939	ATG	TAA	6269-7207	7	6268-7206	7	6268-7206	7
tRNA-Pro	H	68			7215-7282	2	7214-7281	2	7214-7281	2
ND6	H	513	ATG	TAG	7285-7797	9	7284-7796	9	7284-7796	9
CYTB	H	1140	ATG	TAA	7807-8946	17	7806-8945	18	7806-8945	18
tRNA-Ser2	H	68			8964-9031	5	8964-9031	5	8964-9031	5
tRNA-Thr	L	70/71[3]			9037-9106	8	9037-9106	8	9037-9107	8
ND4L	H	297	ATG	TAG	9115-9411	-7	9115-9411	-7	9116-9412	-7
ND4	H	1371	ATG	TAG/TAA[4]	9405-10775	9	9405-10775	9	9406-10776	8
tRNA-His	H	66			10785-10850	1	10785-10850	1	10785-10850	1
ND5	H	1719	ATG	TAA	10852-12570	23	10852-12570	21	10852-12570	21
tRNA-Phe	H	69			12594-12662	0	12592-12660	0	12592-12660	0
putative CR		1549[5]			12663-14211	0	12661-13729	0	12661-13747	0
COX3	H	780	ATG	TAA	14212-14991	33	13730-14509	33	13748-14527	33
tRNA-Lys	H	73			15025-15097	11	14543-14615	5	14561-14633	6
tRNA-Ala	H	67			15109-15175	1	14621-14687	1	14640-14706	1
tRNA-Arg	H	69			15177-15245	5	14689-14757	5	14708-14776	5
tRNA-Asn	H	67			15251-15317	13	14763-14829	14	14782-14848	13
tRNA-Ile	H	69			15331-15399	4	14844-14912	4	14862-14930	4
ND3	H	354	ATG	TAA	15404-15757	-1	14917-15270	-1	14935-15288	-1
tRNA-Ser1	H	67			15757-15823	0	15270-15336	0	15288-15354	0
ND2	H	1059	ATG	TAA	15824-16882	5	15337-16395	5	15355-16413	5

[1]Values are relative to the next gene; negative values represent overlapping nucleotides;

[2]Length for L. saxatilis: 895bp; for L. obtusata and L. fabalis: 894bp;

[3]Length for L. saxatilis and L. obtusata: 70bp; for L. fabalis: 71bp;

[4]Stop codon for L. saxatilis: TAG; for L. obtusata and L. fabalis: TAA;

[5]Length for L. saxatilis (single complete sequence in the dataset)

Supplementary Tables

Supplementary Table 1. Nucleotide composition of the L. saxatilis (sax), L. obtusata (obt) and L. fabalis (fab) mitogenomes. CR: control region; PCGs: protein-coding genes; rRNAs: ribosomal RNA genes.

Species	Region	Length (bp)	AT%	A%	T%	G%	C%
sax	**Full sequence**	16887	66.9	30.4	36.5	14.1	18.9
obt		16300	66.3	29.9	36.4	14.7	19.1
fab		16318	66.2	29.9	36.3	14.9	18.9
		Average	66.5	30.1	36.4	14.6	19.0
sax	**Full sequence without repetitive region of CR**	15927	66.2	29.6	36.6	14.8	19.0
obt		15920	66.3	29.6	36.7	14.8	18.9
fab			66.2	29.5	36.7	14.9	19.0
		Average	66.2	29.5	36.7	14.8	19.0
sax	**Full sequence without CR**	15338	66.1	29.3	36.8	15.0	18.9
obt		15331	66.2	29.3	36.8	15.0	18.8
fab			66.1	29.3	36.8	15.1	18.8
		Average	66.1	29.3	36.8	15.1	18.8
sax	**CR without repetitive region**	589	68.3	36.0	32.3	8.8	22.9
obt			68.6	35.3	33.3	9.7	21.7
fab			68.6	35.0	33.6	9.3	22.1
		Average	68.5	35.4	33.1	9.3	22.2
sax	**PCGs**	11250	65.0	27.1	38.0	14.9	20.1
obt			65.1	27.1	38.0	14.9	20.0
fab			64.9	27.0	37.9	15.0	20.0
		Average	65.0	27.1	38.0	14.9	20.0
sax	**rRNAs**	2307	69.2	36.3	32.9	16.0	14.8
obt			69.1	36.2	32.9	16.2	14.8
fab			69.3	36.4	32.8	16.0	14.8
		Average	69.2	36.3	32.9	16.0	14.8

Supplementary Table 2. Littorinimorpha mitogenomes available in GenBank by August 2016 besides Littorina. For each species, taxonomic position at the level of superfamily, together with GenBank accession number, length of the full sequence and nucleotide composition are indicated.

Species	Superfamily*	GenBank a.n.	Length (bp)	AT%	A%	T%	G%	C%
Ceraesignum maximum	Vermetoidea	NC_014583	15 578	59.4	22.6	36.8	25.4	15.2
Cymatium parthenopeum	Tonnoidea	NC_013247	15 270	69.1	30.9	38.2	16.0	14.9
Dendropoma gregarium	Vermetoidea	NC_014580	15 641	60.3	24.7	35.6	22.5	17.1
Eualetes tulipa	Vermetoidea	NC_014585	15 078	62.2	26.5	35.7	22.4	15.5
Galeodea echinophora	Tonnoidea	NC_028003	15 388	70.9	32.1	38.8	14.5	14.5
Naticarius hebraeus	Naticoidea	NC_028002	15 384	72.7	31.8	40.9	14.7	12.7
Oncomelania hupensis	Truncatelloidea	NC_012899	15 182	67.3	30.0	37.3	16.7	16.0
Oncomelania hupensis hupensis	Truncatelloidea	NC_013073	15 186	67.3	29.9	37.4	16.7	15.9
Oncomelania hupesnsis robertsoni	Truncatelloidea	NC_013187	15 191	67.2	29.6	37.6	16.9	15.9
Potamopyrgus antipodarum	Truncatelloidea	NC_020790	15 110	66.0	28.6	37.4	17.2	16.8
Potamopyrgus estuarinus	Truncatelloidea	NC_021595	15 120	66.3	28.3	38.0	17.6	16.2
Strombus gigas	Stromboidea	NC_024932	15 461	65.8	28.7	37.1	17.6	16.6
Thylacodes squamigerus	Vermetoidea	NC_014588	15 544	60.6	25.6	35.0	20.9	18.4
Tricula hortensis	Truncatelloidea	NC_013833	15 179	73.0	32.5	40.5	14.3	12.7

*according to GenBank

Additional files for Supplementary Material Online:

File name: Littorina_spp_mitogenome_ClustalW_alignment.fas

File format: FASTA

Description: Mitogenome alignment of L. saxatilis, L. obtusata and L. fabalis sequences based on ClustalW, arranged as in Table 2 (from COX1 to ND2). The repetitive region of the CR is not included because it was not considered for divergence estimates, but it will be located between positions 12692 and 12693 of the current alignment.