Cah. Biol. Mar. (2011) 52 :

Possible role of a mitochondrial genome rearrangement in maintaining the spatial segregation of two cryptic species of the *Lessonia nigrescens* species complex

Florence TELLIER[1,2,3,4], Sylvain FAUGERON[3] and Myriam VALERO[1,2]

(1) *UPMC Univ Paris 6, UMR 7144, Equipe BEDIM, LIA DIAMS, Station Biologique de Roscoff, Place Georges Teissier, B.P. 74, 29682 Roscoff cedex, France*

(2) *CNRS, UMR 7144, Equipe BEDIM, LIA DIAMS, Station Biologique de Roscoff, Place Georges Teissier, B.P. 74, 29682 Roscoff cedex, France*

(3) *Center for Advanced Studies in Ecology and Biodiversity, LIA DIAMS, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile*

(4) *Facultad de Ciencias del Mar, Universidad Católica del Norte & CEAZA, Larrondo 1281, Coquimbo, Chile*

Fax: ++56-51-209812. E-mail: ftellier@gmail.com

Abstract: In numerous taxa, studies have reported the co-occurrence of several copies for a single mitochondrial marker (heteroplasmy), leading to incorrect phylogenetic inferences if not detected. While this phenomenon has been widely investigated in terrestrial taxa, it remains largely unexplored in marine algae. Here we report the detection of heteroplasmy in the *Lessonia nigrescens* species complex. This work aimed to identify the distinct fragments and to determine their geographic extent in the distribution range of two kelp species. Using phylogenetic reconstructions of a mitochondrial DNA marker (*atp8/trnS* intergenic region), we suggest that a duplication event occurred, either with or without transfer of the copy to the nucleus. The corresponding sequences constituted a new monophyletic clade, distinct from those previously described in *L. nigrescens*, hence discarding the interspecific hybridization and intraspecific biparental transmission hypotheses. The characterization of 652 individuals sampled along 2,500 km of coastline revealed that the duplication was absent in the Northern species, but of high frequency in the northernmost populations of the Southern species. Because the duplication is restricted to the parapatric contact zone between the two species, our findings open new perspectives about the importance of reproductive isolation mechanisms and local adaptation in the origin and persistence of this pattern.

Résumé : Chez de nombreux taxa, des études ont reporté la cooccurrence de plusieurs copies d'un seul marqueur mitochondrial (hétéroplasmie), ce qui, si ce n'est pas détecté, peut mener à des inférences phylogénétiques erronées. Bien que ce phénomène ait été largement étudié chez les taxa terrestres, peu d'informations existent pour les algues marines. Dans cet article, nous présentons la détection d'hétéroplasmie chez le complexe d'espèces *Lessonia nigrescens*. Lors de ce travail, l'objectif était d'identifier les différents fragments, puis de déterminer leur étendue géographique par rapport à l'aire de distribution de deux espèces de kelp. Grâce à des reconstructions phylogénétiques réalisées pour un marqueur d'ADN mitochondrial (région intergénique *atp8/trnS*), nous suggérons qu'un évènement de duplication a été à l'origine du patron observé, que ce soit avec ou sans le transfert de la copie vers le génome nucléaire. Les séquences correspondantes constituent un nouveau clade monophylétique, distinct de ceux décrits au préalable pour *L. nigrescens*, permettant de rejeter

Reçu le 2 novembre 2010 ; accepté après révision le 27 juin 2011.
Received 2 November 2010; accepted in revised form 27 June 2011.

les hypothèses d'hybridation interspécifique et de transmission biparentale intraspécifique. La caractérisation de 562 individus, échantillonnés sur 2500 km de côte, a révélé que la duplication était absente dans l'espèce Nord, mais en fréquence importante dans les populations de l'extrême nord de l'aire de distribution de l'espèce Sud. Du fait que la duplication est restreinte à la zone de contact parapatrique entre les deux espèces, nos résultats ouvrent de nouvelles perspectives sur l'importance des mécanismes d'isolement reproducteur et d'adaptation locale qui sont à l'origine et contribuent au maintien de ce patron.

Keywords: Heteroplasmy ● Kelp ● Mitochondria ● Parapatric distribution ● Range edge ● Speciation

Introduction

Organelle genomes are usually considered as non-recombining and of uniparental inheritance (Avise, 2000; Ballard & Rand, 2005). These features make them ideal for phylogenetic and phylogeographic inferences, for which the only source of variation is mutation. However, several variants of a mitochondrial marker can be observed within a single individual. This phenomenon known as heteroplasmy suggests that other processes may be acting on organelle genomes. In strict terms, heteroplasmy means the co-occurrence of organelles of different origin (e.g. from both male and female gametes). However, the variants may be located within a single mitochondria, in different mitochondria coexisting within the cell, or in distinct genomic compartments (e.g. different copies of the same mitochondrial marker present in the mitochondria and the nucleus). The co-amplification of distinct variants when using a single pair of specific primers for a polymerase chain reaction (PCR) is a commonly encountered difficulty in barcoding studies (see for example Song et al., 2008) and leads to what is known as "apparent heteroplasmy". When the orthology of the coexisting fragments is violated (i.e. when the fragments are evolving independently from each other), wrong inferences on species history are expected.

While some phylogeneticists consider these coexisting fragments as molecular troublemakers, such pattern may inform us on evolutionary processes affecting the species. To explain the origin of the coexisting variants of a mitochondrial marker, two main groups of hypotheses can be drawn: hypotheses implying a duplication event and hypotheses implying the coexistence of distinct mitochondrial variants within a single cell or organism. The first set of hypotheses is focused on gene duplications, where a partial region of a genome has been duplicated, leading to the coexistence of the original allele and one or several copies (the duplicate, located in another region of the genome or close to the original allele). For mitochondrial markers, the original and its copy can be located both within the same mitochondrial genome (Abbott et al., 2005, here named mitochondrial duplication). Another possibility is a transfer of the copy into the nuclear genome (nuclear-encoded mitochondrial sequences, or NUMT, Richly & Leister, 2004, sometimes named pseudogenes in animals, Bensasson et al., 2001). There is little information about the evolutionary consequences of such a duplication of a mitochondrial gene. Studies on duplicated *nuclear* markers (i.e. both original and copy are nuclear) have nevertheless shown that this phenomenon may provide a source of genetic innovation and/or may provoke genetic incompatibilities between genomes. The acquisition of new functions (Bridges, 1936, Ohno, 1970) can confer a selective advantage in particular environments (e.g. the insecticide resistance in mosquito: Raymond et al., 1991). Also, numerous studies have shown the role of *nuclear* duplications in processes of speciation. In fact, duplications frequently act as a postzygotic reproductive isolation barrier (e.g. chromosomic rearrangement, polyploidization: Rieseberg, 2001, Coyne & Orr, 2004, Rieseberg & Willis, 2007) between closely related species. In contrast, NUMTs and gene duplication within the mitochondrial genome have been poorly studied in the context of speciation. Nevertheless, it is highly plausible that a differential loss of function of the duplicated genes – if expressed – would potentially contribute to the species divergence and/or to species specific adaptation processes. The probability that a NUMT and/or the mitochondrial copy remain functional or acquire a new function is largely dependent on taxa and on the substitutions rates of the two genomic compartments. In animals, the difference of genetic codes between the mitochondria and the nucleus is likely to be the main limitation to NUMT expression (Adams & Palmer, 2003). In contrast, an important number of functional NUMTs has been described in plants (Adams & Palmer, 2003) and mainly concerns genes coding for ribosomal proteins. NUMTs accumulation is probably a continuous process in the evolutionary history of species, with transfer events

mostly independent to each others (e.g. in humans: Gherman et al., 2007, in plants: Palmer et al., 2000). NUMTs may present variation of frequency among species, but also among populations within a single species (Song et al., 2008).

The second set of hypotheses concerns the coexistence of distinct mitochondrial genomes within the cell (true heteroplasmy), resulting from a biparental mitochondrial inheritance (see for review Xu, 2005). When the heteroplasmic individual is the result of crossing between closely related populations, the high similarity of sequence between paternal and maternal mtDNA complicates the heteroplasmy detection, but induces little wrong phylogenetic inference. Interspecific heteroplasmy is easier to detect, as the two mitochondrial sequences are divergent. It has been reported in numerous taxa such as fungi, invertebrates, mammals, birds and plants (see for example: Wagner et al., 1991, Xu, 2005). True heteroplasmy has been frequently reported in hybrid zones between closely related species, in animal and plant taxa (Rokas et al., 2003, Barr et al., 2005) and also in a single algal genus (*Fucus*: Hoarau et al., 2009). When the reproductive isolation mechanisms are incomplete, this unusual biparental inheritance in hybrids seems to be due to a breakdown of the mechanisms that recognize and eliminate the paternal mtDNA in the egg. Therefore, true heteroplasmy is expected to be restricted to hybrid zones and to be transitory: the processes of lineage sorting and genetic drift during the uniparental transmission of the mitochondrial genome will eliminate it rapidly (Rokas et al., 2003). Despite of this transitory state, it may have strong evolutionary consequences, because it is an opportunity for recombination between divergent mitochondrial genomes, leading to the creation of new haplotypes by crossing-over (Rokas et al., 2003). It is also a potential clue for disentangling the processes associated to speciation in secondary contact zones or in peripatric areas where hybrids can be traced. For example, introgression events, the observation of a mitochondrial genome from one species in the nuclear background of another species, may be the results of past heteroplasmy (see for example in algae: Neiva et al., 2010).

Here we report the co-amplification by PCR of several variants of a mitochondrial fragment in the species complex *Lessonia nigrescens* Bory (1826) (Phaeophyceae, Laminariales, also known as kelps). This model taxon includes two closely related species, recently described based on multi-gene phylogenies (Tellier et al., 2009, see also for review: Tellier et al., this issue). Among other characteristics, the two species differ by the size of the intergenic spacer *atp8/trnS*, a mitochondrial marker used in phylogenetic and phylogeographic studies (Voisin et al., 2005; Tellier et al., 2009). Each fragment size is unique to one of the cryptic species and thus a fast determination of the species an individual belongs to may be performed through electrophoresis on agarose gel, without sequencing. Nevertheless, we detected in some cases the co-amplification of several fragments by PCR, with differences in size among fragments. These observations raised new questions regarding the origin of this apparent heteroplasmy: is it the consequence of recent or of ancient hybridization processes? In oogamous Phaeophyceae, maternal inheritance of mitochondria and chloroplast seems to be the rule (Motomura, 1990, Motomura et al., 2010). Heteroplasmy (both intra- and inter-specific) has been reported only for the *Fucus* genus (Coyer et al., 2004, Hoarau et al., 2009), but there is currently no report of pseudo-heteroplasmy (NUMT nor mitochondrial duplication).

Through the analysis of sequence and phylogenetic localization of the co-amplified fragments identified in the *L. nigrescens* cryptic species, we tested the following hypotheses regarding the origin of these co-amplified fragments: (*i*) an intraspecific heteroplasmy, involving fragments of similar sequences; (*ii*) an interspecific heteroplasmy, as the result of a unique or recurrent event of hybridization, involving mitochondria from both cryptic *L. nigrescens* species; (*iii*) a pseudo-heteroplasmy, i.e. the presence of a paralogous copy of the target fragment, localized in the mitochondria or transferred into the nucleus. In addition, we checked for two additional sources of apparent heteroplasmy; (*iv*) plurigenotypic organisms, or chimeras, resulting from the fusion of tissues from distinct zygotes, a phenomenon reported as "coalescence" in marine algae (Santelices et al., 1999; in red seaweeds: Santelices et al., 1996, in green seaweeds: González & Santelices, 2008, and in kelp: Wernberg, 2005) – in this case we expected an intra-individual polymorphism for the target fragment; and (*v*) a cross-amplification of DNA from other species, such as parasites or epi/endophytes (e.g. Lane & Saunders, 2005). In addition, we completed our study by determining the geographic extent of this apparent heteroplasmy and by identifying its frequency at the population-level. A particular sampling effort was done in the contact zone between the species, where hybridization is more likely to occur.

Material and Methods

Model system

As the two cryptic species of *Lessonia nigrescens* are awaiting formal taxonomic description, they are currently distinguished by their geographic distribution along the Chilean coast: the Northern species is found north of 30°S of latitude and the Southern species is located south of 29°S

(Fig. 1). These species have been defined based on multi-genes phylogenies (Tellier et al., 2009) and a complete absence of interspecific gene flow, at least in the contact zone of Aceituno-Choros (Fig. 1, Tellier et al., in press). In addition, differential ecological responses to physiological stresses have been detected (Oppliger et al., 2011, Tellier et al., 2011).

Both species are engineer species, playing a major ecological role by structuring the lower intertidal ecosystem (Vásquez, 2008). In addition, the two cryptic species of *L. nigrescens* are commercially exploited for alginate extraction (Bixter & Porse, in press), with a maximum of harvesting pressure located on the transition zone (29-31°S), i.e. at the range edges of the species (Vásquez, 2008, Tellier et al., 2011).

DNA extraction, amplification and gel migration of PCR products.

Excised thallus tissue was dried in silica gel before DNA analysis. Total DNA was extracted using the following protocol (adapted from Martinez et al., 2003). DNA was extracted from 50 µL of finely ground tissues. This volume was mixed with equal volume of Poly Vinyl Pyrrolidona (PVP) and then extracted with 700 µL of CTAB buffer (3%) in Tris-HCl 1M, pH 8.0, NaCl 1.4 M, EDTA 20mM. The mix was agitated for one hour at ambient temperature, then centrifuged at 14,000 rpm for 30 min. Supernatant was pipeted and mixed with 700 µL of Chlorophorm:Isoamilic Alcohol (24:1) during 1 min. After centrifugation during 20 min at 14,000 rpm at -4°C, the supernatant was precipitated in 0.75 volumes of 100% isopropanol. Then the solution was centrifuged for 14 min at 14,000 rpm at 4°C and the

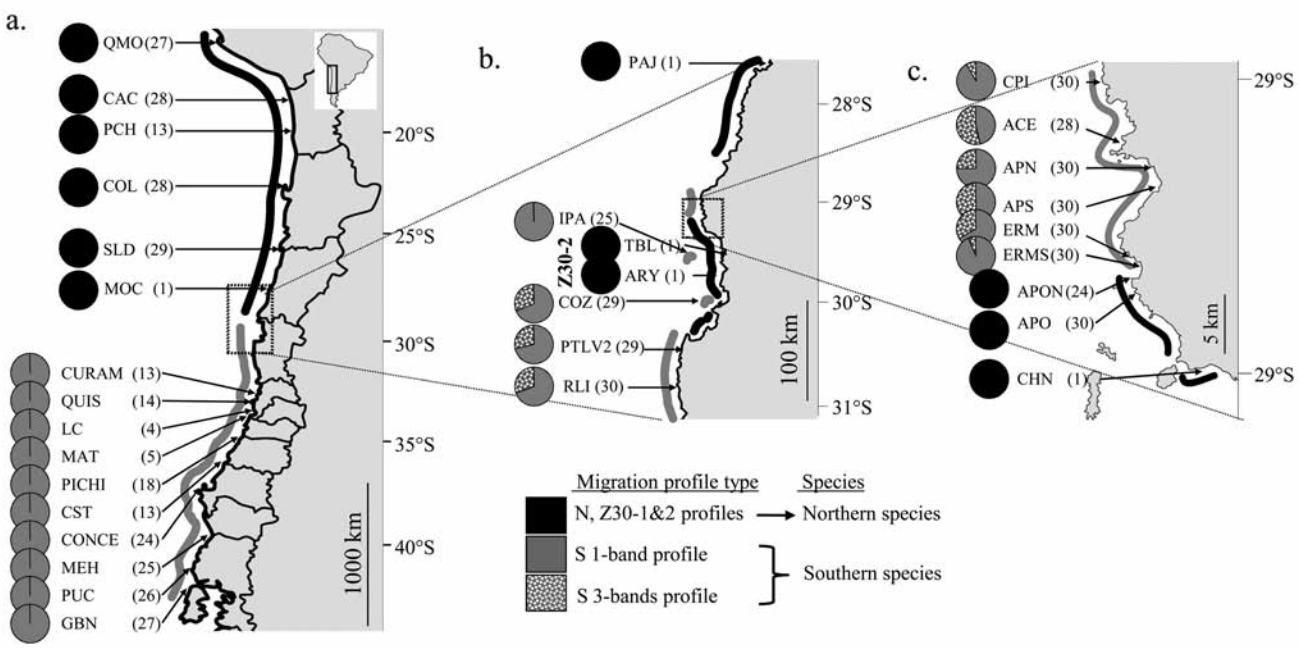


Figure 1. *Lessonia nigrescens*. Geographic distribution of the Northern and Southern species of and distribution of the migration profiles. **A.** At the scale of Chilean coasts. **B.** At the scale of the transition zone (28-31°S). **C.** At the scale of the Aceituno-Choros contact region (29°S). The color of the coastline indicates the distribution of the cryptic species according to Tellier et al. (2009, in press): in grey the Southern species and in black the Northern species. For each sampling site, the number of analysed individuals is indicated in parenthesis and the pie-chart indicates the proportion of individuals belonging to the three major migration profile types: S 1-band and S 3-bands for the Southern species and N/Z30-1&2 for the Northern species (respectively: N profile for populations from QMO to MOC, Z30-1 for PAJ, Z30-2 for others).

Figure 1. *Lessonia nigrescens*. Distribution géographique de l'espèce Nord et de l'espèce Sud et distribution des profils de migration. **A.** A l'échelle de la côte chilienne. **B.** A l'échelle de la zone de transition (28-31°S). **C.** A l'échelle de la région de contact Aceituno-Choros (29°S). La couleur du trait de côte indique la distribution des espèces cryptiques selon Tellier et al. (2009, sous presse) : en gris l'espèce Sud et en noir l'espèce Nord. Pour chacun des sites d'échantillonnage, le nombre d'individus analysés est indiqué entre parenthèses et les secteurs indiquent la proportion d'individus appartenant aux trois principaux types de profil de migration : S 1-bande et S 3-bandes pour l'espèce Sud et N/Z30-1&2 pour l'espèce Nord (correspondant respectivement au profil N pour les sites compris entre QMO et MOC, au profil Z30-1 pour le site PAJ et Z30-2 pour les autres sites).

DNA pellet washed in alcohol 70% prior to drying. The precipitated DNA pellet was diluted in 60 µL of MilliQ water and quantified by NanoDrop (NanoDrop Technologies Wilmington, Delaware, USA).

The mitochondrial marker *atp8/trnS* was amplified using primers designed by Engel et al. (2008) in highly conserved coding regions flanking the spacer: atp8-trnS-F (3' end of *atp8*, 5'-TGTACGTTTCATATTACCTTCTTTAGC-3') and atp8-trnS-R (5' end of *trnS*, 5'-TAGCAAACCAAG-GCTTTCAAC-3') primers. We used the same conditions for PCR and program as Voisin et al. (2005). PCRs were carried out in 20 µL containing 5 µL of sample DNA diluted at 1.6 ng.µL^{-1}, 0.1 mg.mL^{-1} BSA, 2.5 mM $MgCl_2$, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 200 µM dNTPs, 250 nM each primer, 0.5 unit DNA polymerase. After an initial denaturation step (95°C, 5 min), touchdown PCR was carried out for five cycles of 30 s at 95°C, 30 s at 60°C reduced by 1°C each subsequent cycle, and 45 s at 72°C, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s.

According to the complete mitochondrial genome sequence obtained for the kelp *Laminaria digitata* by Oudot-LeSecq et al. (2002), this marker is an intergenic spacer, approximately 244 bp long, located in the mitochondrial DNA between the *atp8* gene and the *trnS* gene. The portion of *atp8/trnS* amplified here corresponded to fragments ranging from 120 bp to 155 bp, as follow (according to Tellier et al., 2009): (*i*) 120 bp in individuals from the Southern species, (*ii*) either 128 bp or 136 bp in individuals from the Northern species within part of its distribution range named the Transition Zone (27-30°S) and (*iii*) 155 bp for individuals of the Northern species from the northern part of its distribution range (12-27°S). Here we named these four fragment sizes respectively as: S, Z30-1, Z30-2 and N fragments. In addition, in case of the co-amplification of more than one fragment, we precised the number of bands in the migration profile (e.g. S 1-band and S 3-bands profile when obtaining 1 or 3 bands for an individual of the Southern species, respectively).

Migration profiles of the PCR products were determined on 2% agarose gels using individuals previously sequenced as positive controls (Tellier et al., 2009). To determine the geographic extent of the observed patterns, we analyzed two sets of individuals: (*i*) 402 individuals covering the entire species' ranges and (*ii*) 250 individuals from the Aceituno-Choros region, one of the parapatric contact zones (Tellier et al., in press, see also for review: Tellier et al., 2011). Sampling information is presented in Fig. 1 (see also Tellier et al., 2009, in press for details).

Fragments isolation and sequencing

Two complementary approaches were used to isolate each band detected and then identify the nature of the fragments. First, we separated the fragments through gel electrophoresis and subsequent sequencing. Second, we cloned and sequenced a subset of individuals. These two approaches were necessary, because of the difficulties each method raised: a low quantity of PCR product in the first method and a differential incorporation of the three fragments within the bacteria used for cloning (see Results).

The first approach consisted in picking up directly the desired fragment: we first ensured a complete segregation of the fragments by migration of the PCR product on a 2%-agarose gel and then we picked up the band of interest using a scalpel under UV-light. A total of 75 individuals were analyzed using this method. PCR products were purified using Nucleospin kits (Machery-Nagel, Düren, Germany) and sequenced on an ABI PRISM© 3100 Automated DNA Sequencer (Applied Biosystems, Foster city, CA, USA).

For the second approach, cloning was performed on four individuals from the ACE population - the region of highest prevalence of the multi-bands profile (Fig. 2; see Results): ACE-A34, ACE-A35, ACE-B3 and ACE-B83. We used the pGEM-T© Vector System (Promega, Madison, WI, USA) following the manufacturer's protocol. Up to five clones were sequenced per individual.

Sequences alignment, polymorphism and phylogenetic reconstructions

Sequences were edited using Chromas v 2.01 (McCarthy, 1997) and were submitted to a BLAST analysis in GenBank (BLASTN 2.2.24+, Altschul et al., 1997). The obtained sequences were aligned with our previously published dataset (Tellier et al., 2009), which included haplotypes from both *L. nigrescens* species and from three other species of the *Lessonia* genus (GenBank accession Nos. EU652976-EU652997, FJ410103-FJ410116, FJ410128 and FJ410130). The multiple sequences alignment was constructed using Multalin (Corpet, 1988) and considered 13 bp of the 3' end of the *atp8* gene, the *atp8/trnS* intergenic region and 23 bp of the 5' end of the *trnS* gene. Indels were treated in two ways: excluding indel sites prior to analysis, and considering indels as missing data, thus considering the possibility for both substitution and indel polymorphisms within a single site. Indels may be informative, but currently there is no consensus on the way of treating them statistically (see Simmons & Ochoterena, 2000). This is particularly true for stretches of indel sites, in which case treating each indel site as evolving separately is unlikely to be a parsimonious method.

Phylogenetic reconstructions were obtained for each alignment by Maximum Likelihood (ML) method and Bayesian inference (BI) using PAUP v 4.0b10 (Swofford,

2002) and MrBayes v 3.1.2 (Huelsenbeck & Ronquist, 2001) respectively; the *L. vadosa* haplotype was used as outgroup. All phylogenetic parameters followed Tellier et al. (2009), except that 500 bootstraps replicates which were done for the estimation of nodal supports of the ML trees.

Results

Geographic extent of the migration profiles

PCR products of all individuals from Northern species (184 individuals from 12 populations) presented a unique band pattern after migration on agarose (named here the profiles N, Z30-1 or Z30-2, depending on the fragment size, Fig. 2), and the fragment size corresponded to the expected fragment size for this species, according to Tellier et al. (2009). For the Southern species, we detected two distinct migration profiles (Fig. 2): (*i*) a unique band profile ("S 1-band profile", 391 individuals, distributed over the 20 populations analyzed for that species), which size corresponds to the expected fragment size for this species, and (*ii*) a profile with three fragments of different sizes (S 3-band profile, in 77 individuals from eight populations over the 20 populations of the Southern species). The smallest fragment was of similar size to the fragment detected in the S 1-band profile.

The S 3-band profile showed a particular geographic distribution (Fig. 1). First, it was restricted to the northern-most populations of the Southern species, between 29°01'S and 30°44'S, covering ~300 km of coastline. Second, its frequency was highly variable even between closely adjacent populations: for example, the CPI and ACE sites, separated by only 7.4 km of coastline, showed frequencies of 0.066 and 0.536 respectively (Fig. 1).

Fragments identification

The smallest band of the S 3-band pattern corresponded to known haplotypes of the Southern species, in all the 75 individuals for which sequences were obtained. These haplotypes were identical to those yet observed in S 1-band PCR products (Tellier et al., 2009). Despite our efforts, no sequence could be obtained for the largest fragment. Sequences for the fragment of intermediate size have been obtained either by sequencing after gel separation of fragments (three individuals) and/or after cloning (four individuals, total of 12 sequenced clones). Over the 15 sequences obtained for this fragment, we detected five haplotypes, named atpX haplotypes (atpX1 to atpX5, GenBank Accession Numbers: HQ291067-HQ291071), differing by four polymorphic sites. Excepting for the individuals included in the present study, we never have detected these haplotypes in our previous analysis, done over more than 1,000 individuals (Tellier et al., 2009).

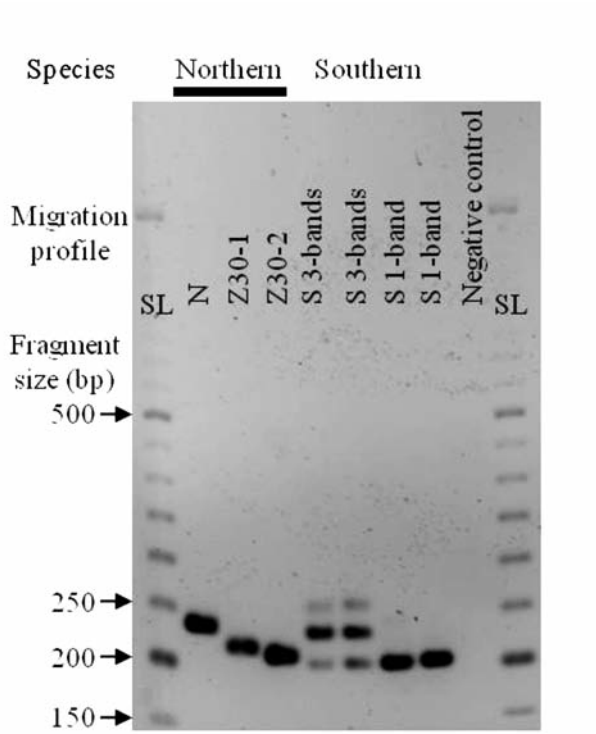


Figure 2. *Lessonia nigrescens*. Types of migration profiles obtained when migrating the PCR products of the mitochondrial marker *atp8/trnS* on an agarose gel at 2%. The name of each migration profile is indicated in the figure, as well as the species the individuals belong to. DNAs used as PCR templates are corresponding to the following individuals (from left to right): PAT14, PAJ3, OTO10, ACE-A34, ACE-B82, PUC3, COZ1, and finally a negative control of the PCR. SL: size ladder. bp: size of the ladder bands in base pairs.

Figure 2. *Lessonia nigrescens*. Types de profils de migration obtenus lors de la migration des produits de PCR du marqueur mitochondrial *atp8/trnS* sur gel d'agarose à 2%. Le nom de chaque profil de migration est indiqué sur la figure, ainsi que l'espèce à laquelle les individus appartiennent. Les ADN utilisés correspondent aux individus suivants (de gauche à droite) : PAT14, PAJ3, OTO10, ACE-A34, ACE-B82, PUC3, COZ1 et en dernier, un témoin négatif de la PCR. SL : marqueur de taille, bp : taille en paires de bases des bandes du marqueur de taille.

Surprisingly, despite the clear S 3-band profile of the PCR products used, the cloning method picked up in all cases only the intermediate fragment. Cloning showed the presence of two distinct "atpX" haplotypes within a single individual (ACE-B3).

Sequences analysis and phylogenetic relationships

The BLAST search indicated that the atpX haplotypes were more similar to other *L. nigrescens* sequences than to other kelp sequences (data not shown). Nevertheless, the five atpX haplotypes shared a common indel structure which is

identical to that of the outgoup species *L. trabeculata*, *L. flavicans* and *L. vadosa*, except for a 1-bp long deletion at position 61 (Fig. 3). The atpX haplotypes presented seven substitutions unique to this haplogroup and shared among all of them (Fig. 3, sites at positions 27, 54, 62, 73, 74, 88 and 132). Within the atpX haplogroup, three substitutions were identified (Fig. 3, sites at positions 101, 121 and 149), from which two were private of this haplogroup (not shared with any other sequence).

All phylogenetic reconstruction methods strongly supported the monophyly of the five atpX haplotypes (nodal support of 94-100%, Fig. 4A & B). However, the exact phylogenetic position of the atpX clade remained poorly resolved. In fact, excluding the indel sites revealed a polytomy for three clades: the two clades of the Northern species (North and Z30 clades) and the atpX clade (Fig. 4B). In contrast, when indels were considered as missing data (i.e. including the substitution polymorphism of the indel sites), then the atpX clade was phylogenetically intermediate between the Southern species clade and the

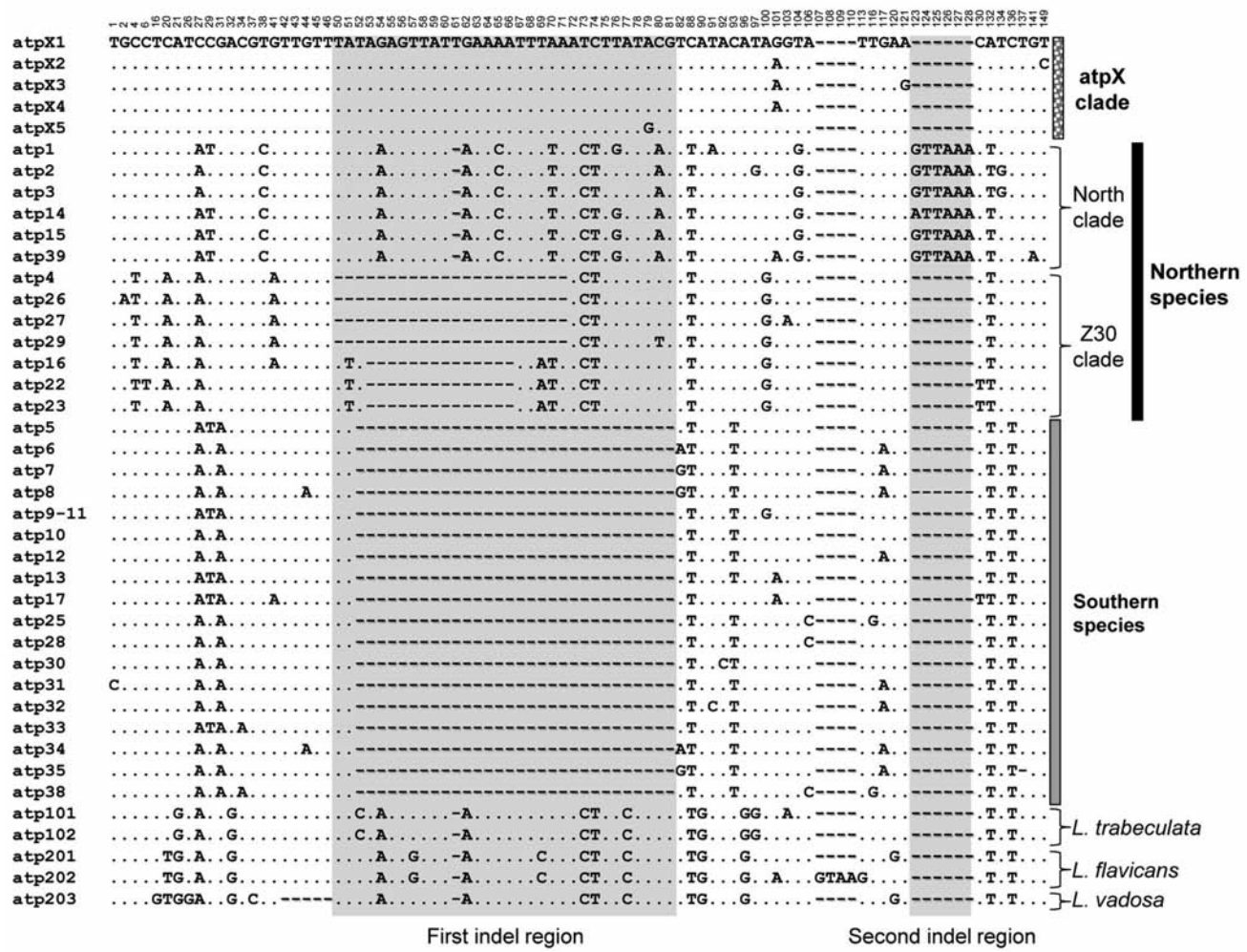
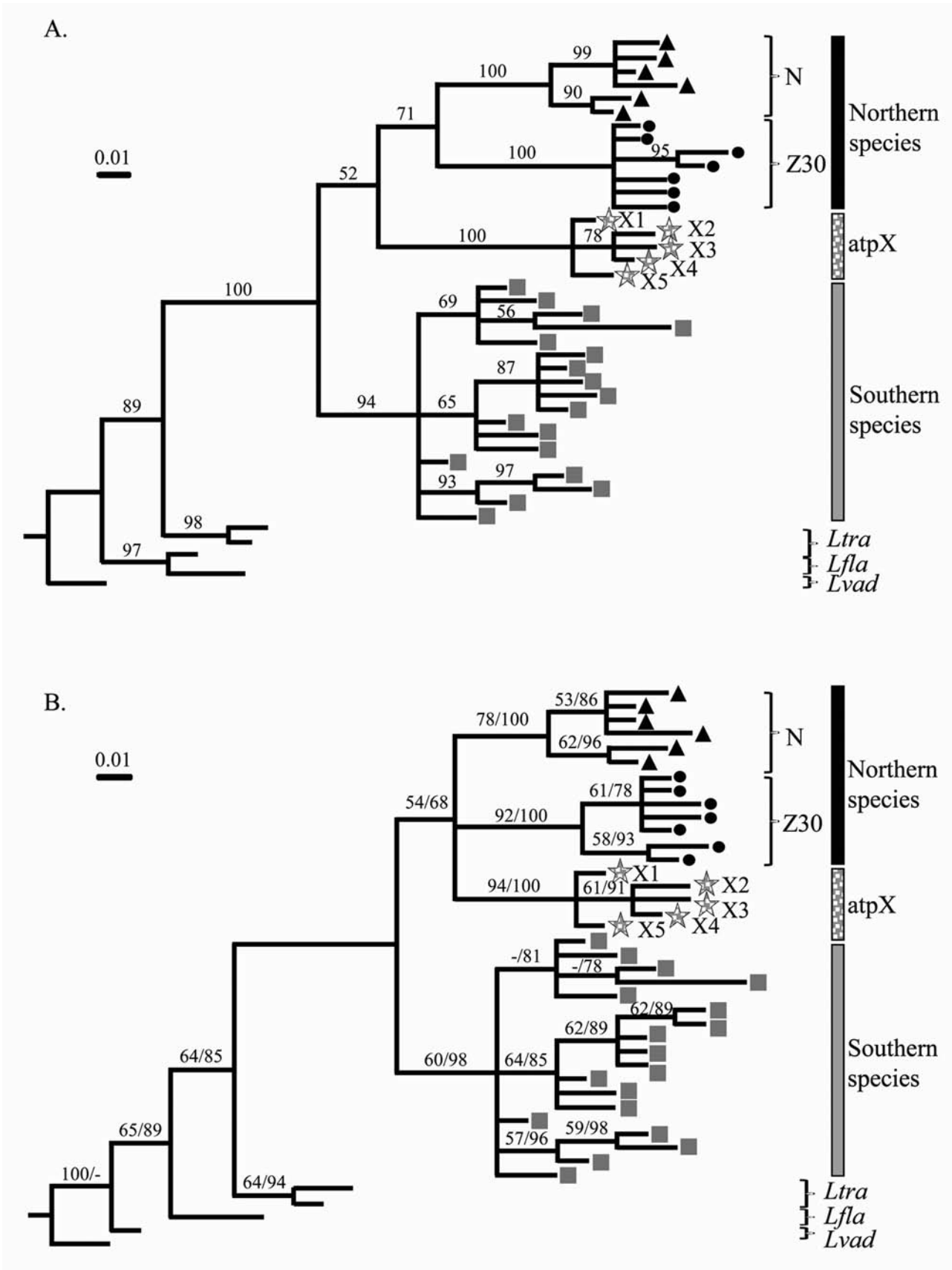


Figure 3. *Lessonia nigrescens*. Alignment of the five *atp8/trnS* haplotypes obtained in the present study (haplotypes atpX1 to atpX5) with the dataset previously obtained and outgroup species (Tellier et al., 2009). Only polymorphic sites are shown. The sequences are grouped by similarity for indel polymorphism, and clades are indicated based on the phylogenetic trees (see Fig. 4). The two indel regions showing polymorphism within the species complex *L. nigrescens* are indicated by shaded area.

Figure 3. *Lessonia nigrescens*. Alignement des cinq haplotypes *atp8/trnS* obtenus dans cette étude (haplotypes atpX1 à atpX2) avec le jeu de données obtenu précédemment pour *L. nigrescens* et les espèces proches (Tellier et al., 2009). Seuls les sites polymorphes sont indiqués. Les séquences sont regroupées en fonction de la similitude du polymorphisme d'insertion-déletion. Les clades sont indiqués en se basant sur les résultats des reconstructions phylogénétiques (voir Fig. 4). Les deux régions d'indel montrant du polymorphisme au sein du complexe d'espèces *L. nigrescens* sont indiquées par des rectangles gris.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Lfla
Lvad

Figure 4. *Lessonia nigrescens*. Bayesian Inference phylogenetic trees for the complete dataset of *atp8/trnS* haplotypes, i.e. those obtained in this study ("atpX") and those previously published (Tellier et al., 2009). **A.** Indels are considered as missing data, support values are presented as Bayesian posterior probabilities. **B.** Indels are excluded from the analysis, support values are indicated as Maximum Likelihood bootstraps and Bayesian posterior probabilities respectively. "-" indicates < 50% support in a particular analysis. The color of the symbols represents the *atp8/trnS* clade of the individuals showing these alleles: black: Northern species, grey boxes: Southern species, dotted grey stars: atpX. In case of Northern species haplotypes, the subclade is indicated by the symbol (circle: Z30 clade, triangle: N clade). Only the position of the new haplotypes (atpX haplotypes) is mentioned here, for more details on other haplotypes, see Tellier et al. (2009). Outgroup species: *L. trabeculata* (*Ltra*), *L. flavicans* (*Lfla*) and *L. vadosa* (*Lvad*).

Figure 4. *Lessonia nigrescens*. Arbres phylogénétiques d'inférence Bayesienne pour le jeu de données complet des haplotypes *atp8/trnS*, c'est-à-dire ceux obtenus dans la présente étude ("atpX") et ceux publiés précédemment (Tellier et al., 2009). **A.** Les indels sont considérés comme des données manquantes, les valeurs de support indiquées correspondent aux probabilités postérieures Bayesiennes. **B.** Les indels sont exclus de l'analyse, les valeurs indiquées correspondent aux valeurs de bootstraps de Maximum de Vraisemblance et aux probabilités postérieures Bayesiennes, respectivement. Le signe "-" indique un support inférieur à 50% dans une analyse donnée. La couleur du symbole représente le clade *atp8/trnS* des individus présentant ces allèles : en noir : l'espèce Nord ; les carrés gris : l'espèce Sud ; les étoiles gris tacheté : atpX. Dans le cas des haplotypes de l'espèce Nord, le sous-clade est indiqué par le symbole (cercle : clade Z30, triangle : clade N). Seule la position des nouveaux haplotypes (haplotypes atpX) est mentionnée ici ; pour plus de détails sur les autres haplotypes, voir Tellier et al. (2009). Abréviations pour les espèces proches : *Ltra* (*L. trabeculata*), *Lfla* (*L. flavicans*) et *Lvad* (*L. vadosa*).

Northern species clades, but with low nodal support (Fig. 4A). The presence of indel sites is likely to be at the origin of the unclear position of the atpX clade.

Discussion

In this study, we have shown the co-amplification of several fragments of a mitochondrial marker in one species of the species complex *Lessonia nigrescens*. One of these fragments corresponded to haplotypes from a previously identified clade of the species complex. In contrast, our study revealed a new haplogroup – named the atpX clade – that always co-amplified with the first fragment. The monophyly of this atpX clade is well-supported, independently of the phylogenetic method used (tree reconstruction methods and indel coding), strongly suggesting that these haplotypes share a common origin. Most surprising, the atpX haplotypes neither corresponded to known haplotypes from the *L. nigrescens* species complex nor to other *Lessonia* species. These particularities of the atpX haplotypes allow us to reject some of the initial hypotheses about the origin and maintenance of the observed pattern. On the other hand, their spatial distribution restricted to the contact zone of the two sibling and parapatric species strongly suggest that they are linked to the speciation process and/or the factors that maintain species segregation. Here, we first discuss on these hypotheses and then focus on other plausible hypotheses for the origin of the atpX haplotypes. Lastly, we conclude on the potential evolutionary significance of the observed pattern, in relation with its peculiar geographic distribution.

Rejected hypotheses about the nature of the apparent heteroplasmy

The atpX haplotypes show a strong divergence with the haplogroups previously identified by a phylogeographic study covering most of the geographic distribution of the *L. nigrescens* species complex (Tellier et al., 2009). However, they neither corresponded to haplotypes of other *Lessonia* species nor to known endophytes such as *Laminariocolax* sp. (Phaeophyceae: Ectocarpale) commonly observed in natural populations of *Lessonia* (Thomas et al., 2009). Therefore, we can discard the hypothesis of a cross-amplification of parasite or epi/endophyte DNA to explain the apparent heteroplasmy observed. Similarly, the atpX haplotypes are more similar to other *L. nigrescens* haplotypes than to those obtained for the outgroup species. Thus, we can reject the hypothesis of a biparental inheritance during a hybridization event between *L. nigrescens* and another *Lessonia* species (particularly the sympatric species *L. trabeculata*). The strong divergence between the clade of atpX haplotypes and those previously described for *L. nigrescens* also evidences that intraspecific biparental inheritance is unlikely to be at the origin of the co-amplification of fragments. It also supports the idea that heteroplasmy does not result from coalescence and fusion of genetically differentiated organisms of the *L. nigrescens* species complex.

Regarding the largest fragment from the S 3-bands profile, our data do not allow for further explanations. Despite our efforts, we were not able to obtain any sequence for this band, probably because of the low concentration of this PCR product compared to the two other products obtained for this profile, and/or mutation

accumulation in the primer annealing sites. Its strong divergence in size suggests however a strong genetic divergence, and the same considerations as above should also apply to this large S3-fragment.

Plausible hypothesis for the origin of atpX fragment

The atpX haplotypes were always found associated to haplotypes of the Southern species in the Transition Zone (Fig. 1). In contrast, for all the individuals from the Northern species and for individuals of the Southern species from outside the Transition Zone, the amplification revealed the presence of a single haplotype (N, Z30-1, Z30-2 or S 1-band pattern). Even considering the more than 1,000 individuals in Tellier et al. (2009), we never detected a multi-band pattern in individuals from the Northern clade. A possible origin of this pattern could be a true heteroplasmy due to bi-parental inheritance in hybrid individuals between the two species. In this particular case, such process would have led to an introgression from Northern species into the Southern species' genome. Because of the high divergence of atpX and haplotypes of the Northern species, such scenario would be possible only by assuming that the hybrid origin is sufficiently ancient so that mutations could have accumulated on the atpX lineage. This is an interesting scenario because it may reveal part of the speciation history between the Northern and the Southern sibling species. However, over long periods, it remains difficult to explain the retention of the introgressed haplotypes in a heteroplasmic genome. As mentioned in introduction, the transitory state of a true heteroplasmy is a consequence of the uniparental inheritance of the mitochondrial genome (i.e. genetic drift and lineage sorting; Rokas et al., 2003). This should have led to a rapid elimination of all but one mitochondrial genome.

For the mitochondrial marker *atp8/trnS*, the phylogeographic study has shown a very low within-population polymorphism, with rarely two coexisting haplotypes within a sampling site (Tellier et al., 2009). This contrasts with atpX, which showed up to four distinct haplotypes in four individuals sampled in a single site. More broadly, the atpX clade is characterized by a high polymorphism of substitution, compared to the other *L. nigrescens* clades. This finding rather supports the hypothesis of a duplication event of part of the mitochondrial genome of the Southern species. Although the coexistence of two distinct atpX sequences within a single individual could be an argument in favor of the nuclear transfer, further studies are needed to discard possible errors commonly occurring when cloning (see for example in seaweeds: Zuccarello et al., 2009) and to determine whether the duplicated sequence is within the mitochondrial genome or has been transferred to the nucleus (NUMT).

In order to determine the extent of the mitochondrial genome that may have been affected by the duplication event, we performed direct sequencing of another mitochondrial marker (*rpl31/rns*, located at ~820 bp from the *atp8/trnS* marker, Oudot-LeSecq et al., 2002) for a subset of six individuals with a S 3-bands pattern for *atp8/trnS* (data not shown). However, no peak ambiguity has been detected for this second mitochondrial marker, suggesting that the duplication event did not involve this region.

The phylogenetic reconstructions did not allow dating the duplication event that originated the atpX clade: depending on the indel treatment, this event appears to be either anterior or posterior to the speciation event between the two cryptic species of *L. nigrescens*. Because of the important divergence between the atpX and the other clades, we can nevertheless suggest that the origin of the atpX is sufficiently ancient to have allowed the accumulation of specific mutations. An alternative hypothesis would be a higher mutation rate for the atpX clade than for the other haplogroups. In fact, differences in mutation rates among paralogous copies are expected, particularly because they are submitted to different selective pressures (Baer et al., 2007). In particular, if duplication with transfer to the nucleus is confirmed, the potential loss of function and the higher effective size due to the diploid nature of the genome should in theory lead to the retention of a higher number of alleles than in the mitochondrial genome, thereby artificially increasing the mutation rate of atpX.

Evolutionary perspectives for the geographic location of the atpX haplogroup

The S 3-band pattern was observed only in the northernmost populations of the Southern species, i.e. between 29°S and 31°S. This region is unique: first, it is the northern edge of the geographic distribution of the Southern species, and second it corresponds to a parapatric edge with close proximity between Southern and Northern species populations.

The reason for the persistence of the atpX sequences in the northernmost populations of the Southern species remains highly speculative. Further studies would be needed to determine if the duplicated region has any evolutionary role. Because the mitochondrial marker studied here is a non-coding intergenic spacer, it is possible that the duplication is very small and that it does not confer any special advantage. Nevertheless, the geographic distribution of the atpX sequences is highly surprising and raises new questions. Does the duplicated region include genes conferring a selective advantage in the particular environmental conditions of the northern limit of the Southern species range? Does the duplicated region contribute to the reproductive isolation detected among the cryptic species? As mentioned earlier, gametic incompatibilities in hybrids have been shown in case of duplicated

nuclear regions, then contributing to reproductive isolation (e.g. chromosomic rearrangement, polyploidization, Coyne & Orr, 2004). But there is currently, to our knowledge, no evidence for a similar role for NUMTs and neither for within-mitochondria duplication.

Lastly, the observation of the atpX variant only within the Southern species populations may be linked to differences in effective size between the two species, with a stronger genetic drift in the Northern species than in the Southern one, at a historical timescale (colonization processes of the northern region) and at a contemporary timescale (differences in disturbance regimes). Indeed, northern Chile is more strongly affected than southern regions by the El Niño Southern Oscillations; this disturbance induces local massive mortality events in Northern species populations (Martínez et al., 2003). These differences in disturbance regimes between species may contribute to a lower population size in the Northern species, compared to the Southern species. On the other side, if peripatric speciation occurred, then a strong founder effect is expected to have accompanied the speciation process. In both cases, the strong genetic drift could have eliminated atpX variants and heteroplasmy in the Northern species.

Perspectives

In order to confirm the duplication suggested by our results and to localize it (either in the nucleus or in the mitochondria), further studies are needed. These would include for example the location of the paralogous copies by fluorescent *in situ* hybridization (FISH) or the study of inheritance patterns in families. Regarding the third (larger) fragment revealed in our study, no information could be obtained, thus its nature remains completely unknown. Nevertheless, our results on the two other fragments raised new evolutionary questions. In the case of a within-mitochondria duplication, did the duplicated region included coding genes? And if so, what are the consequences on the fitness of the individuals? What is the role of the duplication in the reproductive isolation between the two species of *L. nigrescens*? Finally, this study confirmed the singularity of the distribution range edge of the two cryptic species of *L. nigrescens*. Because this region is threatened by the high harvesting pressure, we recommend further consideration when designing management plans and marine reserve networks.

Acknowledgements

We deeply thank P. Ayerdi, J. Correa, C. Daguin, P. Haye, S. A. Krueger, P. Martin, G. Peralta, S. Plouviez, N. Segovia, J. Tapia, the Chango-lab and the LADIMO lab for their help during various stages of the research. We also are grateful to two anonymous reviewers for constructive comments on earlier versions of the manuscript. F. Tellier was supported by a doctoral grant from the French Ministry of Education and Research (MENRT) and by a grant from the "Collège Doctoral Franco-Chilien". This study is part of the research programs FONDECYT 1060493, FONDAP 1501-0001 Program 7, ANR ECOKELP (ANR 06 BDIV 012), Pôle Mer Bretagne, "ARCUS" of the French Ministry of Foreign Affairs, and the Laboratoire International Associé "Dispersal and Adaptation of Marine Species" (LIA DIAMS). Additional supports from FONDECYT 1090742, FONDECYT 3110051, and from PROJECT HYPERLAB, High Yield and Performance Stem Cell Laboratory FP7 – 223011 are also acknowledged.

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