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A preliminary study of genetic diversity in *Cystoseira amentacea* (C. Agardh) Bory var. *stricta* Montagne (Fucales, Phaeophyceae) using random amplified polymorphic DNA

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Cystoseira amentacea var. *stricta* is an endemic key species in the coastal ecosystem of the northern Mediterranean basin. Because it has suffered significant population declines, it has been designated a protected species. Nothing is known about the population genetics of *Cystoseira* spp. Because zygote dispersal is low among the Fucales, low genetic diversity within populations and strong differentiation among populations were predicted. To evaluate genetic variation within and among *C. amentacea* var. *stricta* populations, 54 individuals from four populations were compared using random amplified polymorphic DNA (RAPD) markers. Data were analyzed using analysis of molecular variance (AMOVA) and Bayesian clustering of individuals into populations based on their genotypes (STRUCTURE 2.1 software). Results indicated that genetic variation is high, with most of it distributed within populations (70.93%), and with some genetic mixing among adjacent populations, a result of longer range dispersal. The Lavezzi population (Corsica) is distinct from the other three populations, suggesting isolation by distance. Although more intensive sampling is required, these preliminary results have important implications for conservation policy.

KEY WORDS: *Cystoseira amentacea* var. *stricta*, RAPD markers, Genetic diversity, Mediterranean Sea

INTRODUCTION

In the Mediterranean Sea, canopy forming species in the genus *Cystoseira* are ecosystem engineers, building large and three-dimensional habitats, comparable to the kelp forests in the Pacific Ocean or the laminarian forests in the Atlantic Ocean. *Cystoseira* species can form large communities on rocky substrata, extending from the surface to 100 m depth and hosting a high diversity of animal and algal species (Ballesteros 1990a, b; Bulleri *et al.* 2002). Most *Cystoseira* species are stenoecious (with narrow environmental tolerances), and are thus very sensitive to environmental changes. Several Mediterranean *Cystoseira* populations have declined within the past few decades (Thibaut *et al.* 2005; Serio *et al.* 2006). Explanations proposed for the disappearance of stenoecious *Cystoseira* species include increase of water turbidity, eutrophication, competition with mussels, overgrazing by sea urchins, fishing activities, anchoring (Cormaci *et al.* 2001) or an increase of surface water temperatures due to global climate changes and modifications of the deep circulation of the eastern Mediterranean basin (Serio *et al.* 2006). The key ecological roles of several *Cystoseira* species, plus the increasing threats they are facing, have led the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Council of Europe 1979) to protect five of these species: *C. amentacea* (C. Agardh) Bory [including var. *stricta* Montagne and var. *spicata* (A. Ercegovic) G. Giaccone], *C. mediterranea* Sauvageau, *C. sedoides* (Desfontaines) C. Agardh, *C. spinosa* Sauvageau (including *C. adriatica* Sauvageau) and *C. zosteroides* C. Agardh.

Cystoseira amentacea (C. Agardh) Bory var. *stricta* Montagne is an endemic Mediterranean species confined to the northern coasts from Spain to Greece (Ribera *et al.* 1992). It grows in the upper infralittoral zone on rocky shores, forming monospecific belts in the most exposed sites. It harbours complex communities, providing shelter or habitat for many organisms (Ollivier 1929; Bulleri *et al.* 2002). The major sources of disturbance for *C. amentacea* var. *stricta* are competition with mussel beds, pollution and habitat destruction (Soltan *et al.* 2001; Boudouresque 2004). *Cystoseira amentacea* var. *stricta* is a perennial, monoecious alga, producing sperm and large, nonmotile eggs in hermaphroditic conceptacles. Fertilization is external, occurring within hours of gamete release (Guern 1962). Zygotes are large and negatively buoyant; they rapidly sink, secrete adhesive wall polymers and adhere to surfaces at 12 h postfertilization (Guern 1962). Thus, zygote dispersal is hypothesized to be very limited, as has been shown for other species of the Fucales (Clayton 1990). Such a reproductive pattern is predicted to lead to low genetic diversity within populations and strong differentiation among populations (Coleman & Brawley 2005).

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To address the genetic structure of populations, microsatellites have been developed for several species in the Fucales, including *Fucus serratus* Linnaeus and *F. evanescens* C. Agardh and *Ascophyllum nodosum* (Linnaeus) Le Jolis (Coyer *et al.* 2002; Olsen *et al.* 2002). Coleman & Brawley (2005) used five microsatellite loci to analyze genetic structure and diversity in *F. spiralis* Linnaeus. They found that life history characteristics, such as the perennial nature and longevity of the species and the low buoyancy of its zygotes, are good predictors of genetic diversity but not population structure. *Fucus spiralis* exhibited low genetic diversity within populations as expected but genetic relationships among populations suggested long distance dispersal, probably occurring via drifting algal rafts.

Martinez *et al.* (2005) found that the same microsatellite primers worked for several species within the same kelp order (Laminariales). Six microsatellite markers developed for *A. nodosum* (Olsen *et al.* 2002) and three developed for *F. serratus* (Coyer *et al.* 2002) were tested on *C. amentacea* var. *stricta* DNA. Unfortunately, they gave no amplification products under any polymerase chain reaction (PCR) conditions. The families Fucaceae and Sargassaceae in the Fucales are evidently too divergent to allow the use of the same microsatellite primers. We therefore turned to the multilocus anonymous markers random amplified polymorphic DNA (RAPD).

RAPD has been used for algal taxonomy (Ho *et al.* 1995; Barreiro *et al.* 2006), as well as for assessing impact of disturbances on gene flow among seaweeds (Faugeron *et al.* 2005; Micheli *et al.* 2005), and for the evaluation of genetic diversity of seagrasses (Procaccini *et al.* 1996; Alberto *et al.* 2001; Jover *et al.* 2003) and seaweeds (Miller *et al.* 2000, Engelen *et al.* 2001, Bouza *et al.* 2006). Information on the genetic structure of Mediterranean *Cystoseira* populations is currently lacking. However, such information would be essential for the protection and remediation of this very sensitive species and that is why we conducted this preliminary work. The aims of this study are (1) to test the use of RAPD markers in *Cystoseira amentacea* var. *stricta* diversity analysis; (2) to estimate for the first time the genetic diversity among and within *C. amentacea* var. *stricta* populations at several spatial scales (from 5 to 465 km).

MATERIAL AND METHODS

Sampling

Cystoseira amentacea var. *stricta* was sampled where it formed dense and continuous populations at the sampling stations on the islands of Port-Cros (Var), Bagaud and Gabinière; at St Jean - Cap Ferrat (Alpes-Maritimes); and on the Lavezzi islands (south of Corsica) (Fig. 1, Table 1). The region sampled is under the influence of the Ligurian current (Fig. 1) (Bethoux *et al.* 1988).

At each station, pieces of erect axes from 15 different individuals were sampled. Because of the caespitose habit of the species, samples were selected from axes 1 m away from each other to avoid sampling the same individual twice. After collection, samples were dried and stored in silica gel at room temperature.

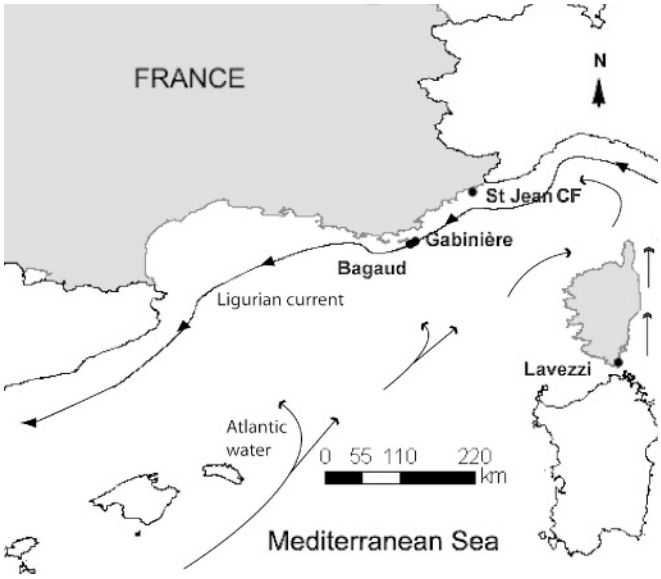

Fig. 1. Location of the sampling stations and main surface currents in the study area.

DNA extraction and amplification

Genomic DNA was extracted from 20 mg dry weight of *C. amentacea* var. *stricta* erect axes, using the QIAGEN 'DNeasy Plant mini kit' according to manufacturer's instructions. DNA was subsequently stored at −20°C. Sixty random 10mer RAPD primers were purchased from OPERON Biotechnologies GmbH (Cologne, Germany; Kits A, B, C) and tested. Four primers, OPA-03, OPB-15, OPB-17 and OPC-02, were selected based on their signal repeatability and variability. Each PCR experiment included one individual (B1) as the positive control and a negative control (without DNA).

The reaction volume (25 µl) contained 5 ng μl^{-1} genomic DNA (1/50 dilution of the original extract), 2 nmol of each dNTP, 25 pmol μl^{-1} of each primer, 2.5 µl of 10× reaction buffer, 2.5 mM of $MgCl_2$ and 1 unit of Platinum® *Taq* DNA Polymerase (Invitrogen Corp., Cergy Pontoise, France). PCR was performed in a Mastercycler thermocycler (Eppendorf) as follows: the initial denaturation at 94°C for 1 min was followed by 45 cycles of 30 s at 94°C, 30 s at 37°C, and 1.5 min at 72°C, with a final elongation at 72°C for 7 min. Amplifications were repeated three times for each sample and only bands found on at least two out of the three replicates were included in the analyses. When band patterns showed no similarity between the three replicates, these individuals were eliminated. Amplifications were performed on 14 individuals from the Bagaud population, 12 individuals from the Gabinière population, 14 from the St Jean - Cap Ferrat population and 14 from the Lavezzi population. Bands with the same molecular weight were treated as identical fragments.

PCR product migration and visualisation

Fragments generated by amplification were separated by size through electrophoresis on 1% agarose gels run in 2× TAE buffer, at 200 mA for 2 h. Bands were visualized by illumination under UV light after staining with ethidium

Table 1. *Cystoseira amentacea* var. *stricta* populations sampled in this study.

Population	GPS position		Distance between populations (km)		
	Latitude (E)	Longitude (N)	B	G	J
Bagaud, Port Cros (B)	6°21′	43°0′			
Gabinière, Port Cros (G)	6°23′	42°59′	5		
St Jean - CF (J)	7°20′	43°41′	115	110	
Omu Mortu, Lavezzi islands, Corsica (M)	9°15′	41°20′	465	460	350

bromide (0.2 μg ml^{-1}). RAPD fragment size was estimated with the 1 kb Plus DNA Ladder (Invitrogen) using the 1Dscan EX 3.1.0 Eval software (Scanalytics Inc.).

Diversity analysis

Since RAPD are dominant markers, we could not detect heterozygosity; each band represents the phenotype at a single biallelic locus. Bands were scored as presence (1) or absence (0) and a matrix of the different RAPD phenotypes was assembled.

Analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was used to elucidate the relationships between groups, populations and populations within groups. The four sampling stations were divided into three groups based on their location: the Port-Cros group, which included two populations, Bagaud and Gabinière; the two other groups each included one population, St Jean - CF and Lavezzi, respectively. AMOVA was performed using Arlequin 3.01 (Excoffier *et al.* 2005). STRUCTURE 2.1 software (Pritchard *et al.* 2000; Pritchard & Wen 2004) was used to estimate the number of genetically homogeneous groups of individuals in our data (i.e. the true number of clusters) by a Bayesian approach. This software determines clusters of individuals on the basis of their genotypes at multiple loci, using a Bayesian approach. The method assumes a model in which there are K populations, each characterized by a set of allele frequencies at each locus. For each individual, the software computes the proportion of the genome that can be assigned to the inferred K populations with a high probability. The model assumes that within populations, the loci are at Hardy-Weinberg and linkage equilibrium. All parameters were set to their default values as suggested in the user's manual (Pritchard & Wen 2004). Independent runs for each value of K (the true number of clusters) from K = 1 to K = 8 were performed (100,000 iterations, no admixture model, as suggested for dominant markers by Pritchard *et al.* 2000). The estimated log posterior probability of data Pr(X/K) for each value of K, which allows the estimation of the likely number of clusters (Pritchard *et al.* 2000), was calculated.

RESULTS

RAPD profiling

The four primers (OPA-03, OPB-15, OPB-17 and OPC-02) used to screen the 54 individuals generated a total of 62 suitable DNA bands, ranging from 300 to 2000 bp. Each population showed different characteristic RAPD profiles, as seen in Fig. 2. No identical genotypes were identified in the same or in different populations. The total number of amplified bands generated by one primer varied from 13 (OPC-02) to 18 (OPB-17), with most of them (98.4%) being polymorphic (Table 2). Eighteen bands out of 62 were present in all four populations (29%), while 18 other bands were population-specific, with four bands unique to Bagaud, three to Gabinière, six to St Jean - CF and five to Lavezzi populations. Twenty-six bands were present in at least two populations.

AMOVA results

Most genetic variation occurred within populations (70.93%). The fixation index (Φ_{st}) was high and significant, at 0.291, indicating a strong genetic differentiation within populations. Of the variation, 11.34% was attributed to among populations-within groups variability, with a significant Φ_{sc} value of 0.138. Although the AMOVA attributed 17.73% of the total variation to regional differentiation, the corresponding Φ_{ct} was not significant (Table 3).

Pairwise F_{st} estimates were all high and significant, ranging from 0.1585 between Bagaud and Gabinière, to 0.3240 between Bagaud and Lavezzi (Table 4). These values appeared to increase with the distance between stations, but data were not sufficient to allow calculation of a correlation coefficient.

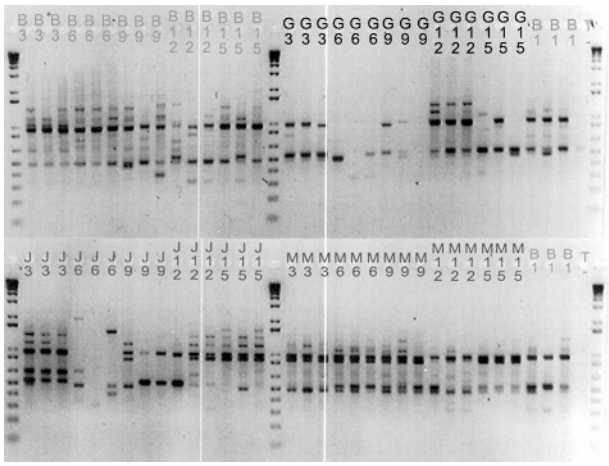

Fig. 2. Examples of RAPD profiles for five individuals of each population for the OPB-17 primer. Three individuals were removed from the analysis according to the nonrepeatability of the signal: G6, G9 and J6. B, Bagaud; G, Gabinière; J, St Jean - CF; M, Lavezzi; B1, positive control.

Table 2. Number of monomorphic (m) and polymorphic (p) sites detected within each population for each primer.

	OPA-03		OPB-15		OPB-17		OPC-02	
Population	m	p	m	p	m	p	m	p
Bagaud (B)	1	6	0	8	1	10	1	8
Gabinière (G)	1	7	0	11	1	8	1	8
St Jean - CF (J)	0	13	0	11	1	11	0	9
Lavezzi (M)	0	10	1	6	1	10	2	8
All populations	0	15	0	16	1	17	0	13

Table 3. AMOVA analysis results for differentiation between the four populations. Three groups were fixed for the analysis: Port-Cros (Bagaud + Gabinière), St Jean - Cap Ferrat and Lavezzi.[1]

Source of variation	df	SS	VC	% Total variance	Fixation indices
Among groups	2	94.061	1.571	17.73	$\Phi_{ct} = 0.177$
Among populations within groups	1	19.291	1.006*	11.34	$\Phi_{sc} = 0.138$
Within populations	53	314.500	6.290*	70.93	$\Phi_{st} = 0.291$
Total	56	427.852	8.867		

[1] df, degrees of freedom; SS, sum of squares; VC, variance components.
* $P < 0.0001$, for 1023 permutations.

Assignation of individuals to populations

Different values for K, the number of clusters of genetically homogenous individuals, were tested. For K values ranging from 2 to 4, each cluster contained at least one individual with more than 50% of its genome represented (Table 5, Fig. 3). On the contrary, for $K \geq 5$, clusters were obtained to which less than 50% of any genome was assigned. For this reason, these values of K were rejected.

At any K value, the Lavezzi population (M) always formed a unique cluster. All Lavezzi individuals were always assigned to a single cluster. For K = 2, populations were segregated according to their location: Bagaud and Gabinière in one cluster, St Jean - CF and Lavezzi in a second. For K = 3, the Bagaud and Gabinière populations formed a single cluster, whereas the Lavezzi population and the St Jean - CF population constituted the second and third clusters respectively. For K = 4, the Lavezzi population formed a single cluster, which included 100% of its individuals; 95% of the genomes of Gabinière individuals were assigned to another cluster; 45% of Bagaud individuals shared the same cluster as Gabinière individuals. Only 55% of Bagaud individuals were assigned to a separate cluster (Table 5, Fig. 3). The highest Ln Pr(X/K) value (i.e. the number of clusters with the greatest likelihood) corresponded to K = 4 (Ln Pr(X/K) = −1114.70), confirming that our separation of samples into four geographical populations was justified.

DISCUSSION

This work represents the first use of molecular markers to characterize genetic diversity in the genus *Cystoseira*. Using RAPD markers, we detected genetic polymorphism in *C. amentacea* var. *stricta* populations, comparable to that observed for other Fucales species (Lu & Williams 1994; Engelen *et al.* 2001).

AMOVA analyses show that genetic diversity is evident among *C. amentacea* var. *stricta* individuals at small (within population) and large (between populations) spatial scales. Most genetic variation is distributed within populations (70.93%), excluding the probability of high levels of inbreeding and/or asexual propagation. Thus, life history characteristics are not good predictors of genetic diversity for this species, as opposed to the case of within-population diversity in *F. spiralis* (Coleman & Brawley 2005). We observed high genetic diversity within populations and among populations within groups, indicating that the four populations are strongly genetically differentiated. Conservation policies must therefore consider each population separately.

Dispersal at distances within 5 km has also been demonstrated. Several individuals of Bagaud were preferentially assigned to Gabinière by the STRUCTURE analysis. This raises the question of dispersal mechanisms. Baez *et al.* (2005) found a U-shaped relationship between species richness and current flow for *Cystoseira* species in

Table 4. Pairwise F_{st} estimates between populations.

	Bagaud (B)	Gabinière (G)	St Jean - CF (J)
Gabinière (G)	0.1585*		
St Jean - CF (J)	0.3114*	0.2134*	
Lavezzi (M)	0.3240*	0.3121*	0.2560*

* $P < 0.001$.

Table 5. Mean percentage of individual genomes assigned to each cluster with STRUCTURE analyses, for K values ranging from 2 to 4. Numbers of individuals per population are given between brackets.

		Given populations[1]			
	Inferred clusters	B (14)	G (12)	J (14)	M (14)
K = 2	1	100	94	6.9	0
	2	0	6	93.1	100
K = 3	1	0	0	9.8	100
	2	0	2.2	77.1	0
	3	100	97.8	13.1	0
K = 4	1	45.3	95.6	20.7	0
	2	54.7	3.7	0	0
	3	0	0	3.9	100
	4	0	0.7	75.4	0

[1] B, Bagaud; G, Gabinière; J, St Jean - CF; M, Lavezzi.

the Mediterranean Sea. They concluded that for low current flows, dispersal of *Cystoseira* spp. was achieved by the sedimentation of small units (single or few-celled stages) whereas for high current flows, it was achieved by large floating thallus fragments. *Cystoseira amentacea* var. *stricta* lacks vesicles, but thallus fragments, mixed with *C. compressa* thallus fragments or *Posidonia oceanica* leaves are often observed along the coast in the drift (personal observation). Drifting fertile thallus fragments of *C. amentacea* var. *stricta* could thus promote zygote or embryo dispersal, as observed for other seaweeds (Coleman & Brawley 2005). Another dispersal mechanism could be seagulls. On Mediterranean shores, breeding females of *Larus cachinnans michaellis* Naumann feed their nestlings with fronds of *Cystoseira* spp., which are rich in vitamin B_1 (Bartoli *et al.* 1997). These birds could disperse fronds bearing embryos.

The AMOVA showed no variability among groups, but this result must be taken with care. First, the analysis included only four populations and second, the lack of significance of Φ_{ct} could be an artefact of the permutation process in AMOVA, since two of our three groups contained only one population. On the other hand, pairwise F_{st} estimates were all significant and increased with distance between populations. Lastly, results of individual assignation to genetic clusters showed that the Lavezzi population (Corsica) was the most differentiated among the four populations, with its individuals always forming a unique cluster (Fig. 3). All these results suggest isolation by distance among *C. amentacea* var. *stricta* populations,

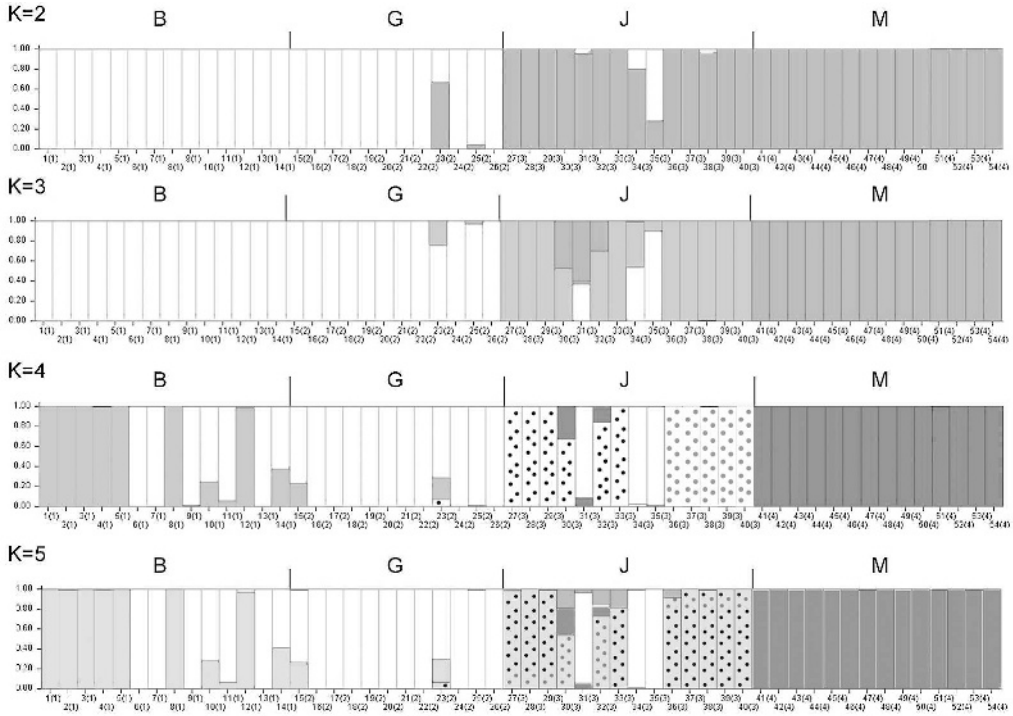

Fig. 3. Bar plots obtained from STRUCTURE analysis for K values (number of clusters) fixed from 2 to 5. The genome of each individual is represented by a single bar. Shadings or symbols filling each bar represent the proportion of individual genome assigned by the software to one of the K clusters. The initial geographic partition of the individuals is reported above the bars (B, Bagaud; G, Gabinière; J, St Jean - CF; M, Lavezzi).

despite a strong local structuring. When K was fixed to 2, the individuals segregated completely into two groups, according to their location: Bagaud and Gabinière in a first cluster, St Jean - Cap Ferrat and Lavezzi in a second cluster (Fig. 3). This conclusion requires further investigation with expanded sampling, especially from the Ligurian coast (northern Italy). If these populations are intermediate between Corsica and the French continent, isolation by distance is likely.

Although *C. amentacea* var. *stricta* is a protected species, its status is not as publicized as *P. oceanica*. It has been overlooked by coastal management programmes and, as a consequence, its habitats have been drastically reduced. To design the most appropriate conservation measures for *C. amentacea* var. *stricta*, extensive population genetic studies throughout the range of *C. amentacea* var. *stricta* in the Mediterranean basin are needed. This study is preliminary, since it was performed only on 54 individuals, with four RAPD markers and four sampling stations. Moreover, because RAPD bands represent dominant markers, their utility in elucidating detailed population structure is limited. The development of species-specific microsatellite loci is necessary to clarify population connectivity in this species.

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