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Molecular forensics in the precious Mediterranean red coral, *Corallium rubrum*: testing DNA extraction and microsatellite genotyping using dried colonies

Jean-Baptiste Ledoux · Didier Aurelle ·
Jean-Pierre Féral · Joaquim Garrabou

Abstract *Corallium rubrum* is an overharvested precious coral submitted to strong environmental pressures. The development of new management tools such as assignment tests is crucial to assess the origin of traded colonies and to reinforce existing regulations in order to ultimately reduce poaching. As a starting point, we test the feasibility of DNA extractions and PCR amplifications of ten microsatellites using dried tissues such as those that can be obtained from traded or seized colonies. We genotype the same ten colonies conserved in alcohol and after five drying periods (from 7 days to 8 months) as well as ten dried colonies coming from a seizure conducted in 2009. Estimating the rate of negative PCRs through time, PCR repeatability and frequencies of null alleles, we demonstrate that dried colonies can be successfully genotyped. This study opens new

avenues for the conservation of *C. rubrum* and other precious corals.

Keywords Poaching · *Corallium rubrum* · Dried tissues · Degraded DNA · DNA extraction · Microsatellites · Genotyping

The Mediterranean red coral, *Corallium rubrum* (L. 1758), is a long-lived octocoral with a slow population dynamics (Linares et al. 2012). Besides environmental pressures resulting in mass mortality events (Garrabou et al. 2009), this precious coral is submitted to overharvesting for its use in jewelry (Bruckner 2009). Included in two international conventions (annex III of the Bern Convention and annex V of the European Union Habitats directive), its harvesting is regulated by national legislations. However, illegal harvesting is expanding and particularly within marine protected areas (Tsounis et al. 2010). Accounting for these pressures, there is a need to reinforce existing regulations in order to reduce poaching. The ability to characterize the origin of traded red coral colonies using molecular forensic methods combining genetic markers and assignment tests (Manel et al. 2002) is therefore crucial. To date, microsatellites are the markers of choice in *C. rubrum* (Costantini and Abbiati 2006; Costantini et al. 2007, 2011; Ledoux et al. 2010a, b; Aurelle et al. 2011) due to the limited genomic resources available in this species combined with the low evolutionary rate of mitochondrial DNA (Calderon et al. 2006) and the lack of information of Internal Transcribed Spacers (Aurelle et al. 2011). As a first step in the development of forensic analyses in the red coral, we thus test DNA extractions and microsatellites genotyping using dried tissues such as those that can be obtained from traded or seized colonies.

J.-B. Ledoux (✉) · J. Garrabou
Institut de Ciències del Mar, CSIC, Passeig Marítim de la
Barceloneta 37-49, 08003 Barcelona, Spain
e-mail: jbbaptiste.ledoux@gmail.com

J. Garrabou
e-mail: garrabou@icm.csic.es

J.-B. Ledoux
CIMAR/CIIMAR, Centro Interdisciplinar de Investigação
Marinha e Ambiental, Universidade do Porto, Rua dos Bragas 177,
4050-123 Porto, Portugal

J.-B. Ledoux · D. Aurelle · J.-P. Féral · J. Garrabou
UMR 7263 IMBE, CNRS, Aix-Marseille University,
13007 Marseille, France
e-mail: didier.aurelle@univ-amu.fr

J.-P. Féral
e-mail: jean-pierre.feral@imbe.fr

Our study was conducted on two samples coming from the field and from a seizure. We sampled ten colonies from Tunel del Dofi (DOF) in Catalonia (Spain, 42.0436°; 3.22644°). Each colony was divided in two parts: one preserved in 96 % alcohol and stored at -80 °C until DNA extraction (reference colony) and the other part conserved at room temperature during 8 months (dried colony). For each colony, total genomic DNA was extracted with a salting out procedure (Miller et al. 1988) after different drying periods: from T-0 (reference colonies) to T-5 (after 8 months of drying) (Table 1a). We also extracted DNA from ten colonies seized in September 2009 (after 25 months of drying) by rangers of the “Generalitat de Catalunya”. DNA suitability was evaluated by PCR amplifications of ten microsatellites following Ledoux et al. (2010a). Electrophoresis of the PCR products was conducted on an ABI 3730 (Applied Biosystems) at the Plateforme Génome Transcriptome de Pierroton (Bordeaux, France). Electrophoregrams were analyzed with STRand (<http://www.vgl.ucdavis.edu/informatics/strand.php>). We assessed the rate of negative PCRs through time as the

number of PCR failure divided by the total number of PCRs over all loci for each drying period. Repeatability was estimated based on DOF samples by computing the rate of errors per reaction for each locus as the number of different genotypes over the six PCRs (T-0 to T-5) for each individual and divided by the total number of PCRs (Pompanon et al. 2005). Frequencies of null alleles were computed for DOF at T-5 and for the seized colonies with FREENA (Chapuis and Estoup 2007) and compared to values from Ledoux et al. (2010a).

We conducted 70 extractions. The amount of tissues used for extractions with dried colonies ranged from 8 to 38 mg. The rate of negative PCRs was not correlated with the length of drying period, the lowest rate (0.07) being observed for T-0 and T-4 and the highest one (0.11) for T-2. Interestingly, this rate was mostly similar for the seized sample (0.12; Table 1a). The rate of error per reactions ranged from 0 for Mic20, Mic22, Mic23 and COR46bis to 0.13 for Mic27 with a mean of 0.04 (Table 1b). These rates were within the range of published values using alcohol conserved (0.004; Hoffman and Amos

Table 1 (a) Rates of negative PCRs for the DOF and the seized samples; (b) The rate of error per reaction is presented for each locus using DOF samples. Mean null alleles frequencies are computed over

DOF at T-5 and the seized samples (present study) and compared to values obtained from Ledoux et al. (2010a)

Rate of negative PCRs			
<i>(a)</i>			
DOF			
T-0 (0 day)	0.07		
T-1 (7 days)	0.06		
T-2 (19 days)	0.11		
T-3 (46 days)	0.08		
T-4 (77 days)	0.07		
T-5 (224 days)	0.08		
Seized colonies (>730 days)	0.12		
Rate of error per PCR		Null alleles frequencies	
		Present study	Ledoux et al. (2010a)
<i>(b)</i>			
Mic13	0.03	0.28	0.24
Mic20	0	0.01	0.07
Mic22	0	0.33	0.37
Mic23	0	0.38	0.4
Mic24	0.07	0.08	0.02
Mic25	0.02	0.11	0.38
Mic26	0.02	0	0.01
Mic27	0.13	0.48	0.16
COR9bis	0.1	0.46	0.4
COR46bis	0	0.17	0.04
Mean (SD)	0.04 (0.05)	0.23 (0.18)	0.21 (0.17)

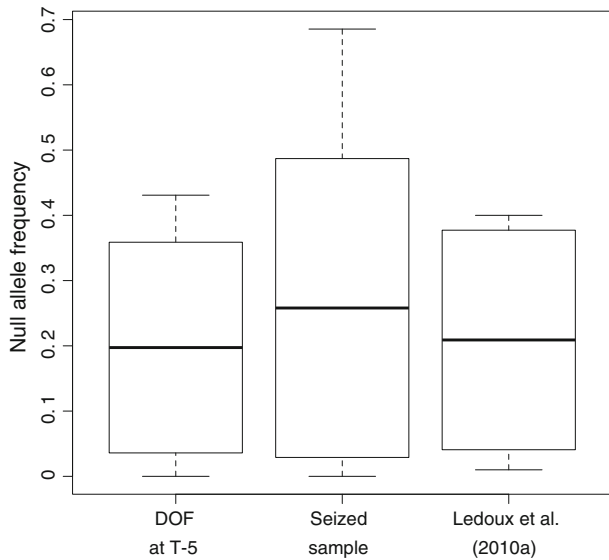


Fig. 1 Boxplots (mean, SD and minimum and maximum values) of null alleles frequencies over the ten microsatellites for DOF at T-5 and the seized samples and obtained from Ledoux et al. (2010a)

2005) or degraded tissues (0.08; Pompanon et al. 2005), suggesting a standard repeatability of the PCRs through time. The frequencies of null alleles for each locus were not different from those previously published except for Cor46bis and Mic27 (Table 1b). It is noteworthy that Mic27 shows a large mean allele size that could explain the differences to the previous studies. Indeed, Sefc et al. (2003) demonstrated a link between PCR failures using DNA from poorly conserved tissues and the amplicon size. When considering all loci, null allele frequencies of the two samples were concordant with published ones in *C. rubrum* (Fig. 1) and in other marine invertebrates (e.g. Polato et al. 2010).

Our study thus validates the use of DNA extracted from dried tissues for microsatellite genotyping in the red coral. Even though Mic27 should be used with caution, we show that PCR reliability was not significantly impacted by putative DNA degradation due to a non-optimal preservation of the tissues. To go further in the development of forensic analyses in the red coral, the efficiency of these microsatellites for assignment tests remains to be tested specially when considering the occurrence of null alleles. Null alleles are very common in numerous taxa (Selkoe and Toonen 2006) and usually have limited impacts on assignment success (Carlsson 2008). Moreover, Manel et al. (2002) demonstrated that this success is correlated with the levels of polymorphism and genetic structure. Interestingly, a high heterozygosity (mean H_e over ten microsatellites and 40 populations = 0.74; Ledoux et al. 2010a) and a strong genetic structure (mean F_{ST} = 0.1 over 760 pairwise comparisons; Ledoux et al. 2010a) characterize red coral

populations. Accordingly, clustering analysis revealed unambiguous genetic clusters in these populations (Ledoux et al. 2010a). Excluding Mic27 should not significantly reduce the assignment success of the remaining microsatellites as the heterozygosity and F_{ST} values stay on the same order of magnitude (mean H_e over nine microsatellites and 40 populations = 0.72; mean pairwise F_{ST} = 0.1 over 760 pairwise comparisons). Therefore, the present study combined with available datasets (Ledoux et al. 2010a; Aurelle et al. 2011) opens new avenues for the conservation of *C. rubrum*. This method should be tested with historical samples and other precious coral species.

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