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Efficiency of Fluorescent Multiplex Polymerase Chain Reactions (PCRs) for Rapid Genotyping of Harbour Porpoises (*Phocoena phocoena*) with 11 Microsatellite Loci

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Abstract

We developed two multiplex sets (*PPH1* and *PPH2*) to amplify 11 polymorphic microsatellite loci previously used in harbour porpoise (*Phocoena phocoena*) studies with only four polymerase chain reactions (PCRs). *PPH1* allows for the amplification of six loci at once, and *PPH2* requires three PCR reactions to amplify five loci. These two multiplex sets were tested on 100 animals from the Belgian coast and the Black Sea. They provided a rapid and efficient genotyping procedure for large-scale population genetic studies.

Key Words: *Phocoena phocoena*, harbour porpoise, cetacean, microsatellites, multiplex-PCR, population genetics

Introduction

Harbour porpoise (*Phocoena phocoena*) are the smallest cetacean, widely distributed in coastal waters of the temperate and sub-arctic northern hemisphere, with a relict population in the Black Sea (e.g., Gaskin, 1984). Throughout their range, harbour porpoises are highly vulnerable to mortality in commercial gillnet fisheries, which may threaten the populations (Jefferson & Curry, 1994). Up to now, the ability to assess fishery impact on harbour porpoises has been limited by a lack of adequate information, and especially the uncertainty about the identity of the populations (Stenson, 2003).

Recent genetic studies on harbour porpoise populations used different sets of microsatellite loci, some specifically designed for the species (Rosel et al., 1999) and others not (Andersen et al., 2001). Unfortunately, the use of different loci among local studies prevents meta-analysis of these results for further wide-scale study. At the same time, for studies requiring genetic characterisation of many individuals at a set of microsatellite loci, time, and cost become serious limiting factors. Multiplexing techniques, which correspond to the amplification of several markers in the same polymerase chain reaction (PCR), have proven to be useful to reduce laboratory manipulations and the consumption of expensive PCR reagents (e.g., Bonnet et al., 2002). The objective of this work was to set up rapid genotyping multiplex PCR kits with existing microsatellites that can be used as a standard for population genetics analyses of harbour porpoise in the field.

Materials and Methods

Sample Collection and DNA Extraction

Harbour porpoise tissues (kidney, muscle, or skin) were obtained from by-catch or stranded animals along the Belgian coast (n = 40) and in the Black Sea (n = 60). The laboratory set-up was performed on samples from five animals and then on the by-catch samples. Total genomic DNA was extracted from tissues preserved in ethanol 95% using DNeasy™ Tissue Kit (Qiagen), following the manufacturer's recommendations.

Microsatellite Markers

To allow calibration between studies, we selected 11 polymorphic microsatellites, seven of 12 analysed by Andersen et al. (2001) and four of eight defined by Rosel et al. (1999) (Table 1; see additional studies referenced there). The choice of the markers allowed multiplex combinations that took into account the capacity of co-amplification with the same PCR conditions, the absence of artifacts resulting from primer dimerization (hypothesis previously tested using the *Amplify 1.2* software;

Table 1. Characteristics of the 11 microsatellite loci for *Phocoena phocoena* and polymorphism statistics of 100 animals from two populations

								Belgian coast			Black Sea		
Microsatellite set	Locus	Ref.	Repeat no.	Primer sequence (5'-3')	PCR	Size range (bp)	Size shift (bp)	A	H_O	H_e	A	H_O	H_e
PPH1	Igf-1	a, b	2	F: FAM-GGGTATTGCTAGCCAGCTGGT R: CATATTTTTCTGCATAACTTGAACCT	1	127-153	1	11	0.87	0.87	11	0.78	0.73
	EV94	c	2	F: FAM-ATCGTATTGGTCCTTTTCTGC R: AATAGATAGTGATGATGATTCACACC	1	195-209	5	6	0.73	0.79	4	0.47	0.43
	PPH110	d	2	F: HEX-ATGAGATAAAATTGCATAGA R: ATCATTAACTGGACTGTAGACCTT	1	105-131	6	11	0.85	0.81	6	0.48	0.45
	PPH104	d	2	F: HEX-CCTGAGGTGTGTAGTCA R: GACCACTCCTTATTTATGG	1	145-177	4	12	0.85	0.88	9	0.66	0.65
	GT011	e	2	F: NED-CATTTTGGGTTGGATCATTC R: GTGGAGACCAGGGATATTG	1	100-136	4	10	0.80	0.81	3	0.47	0.43
	PPH130	d	2	F: NED-CAAGCCCTTACACATATG R: TATTGAGTAAAAGCAATTTTG	1	175-201	6	13	0.90	0.89	8	0.63	0.65
PPH2	PPH137	d	2	F: FAM-CAGGGCGGCCATGTACAGTTGAT R: GAGTTTGGCTCCCTCTCCAG	2	90-136	5	17	0.90	0.89	9	0.73	0.65
	GATA053	f	4	F: FAM-ATTGGCAGTGGCAGGAGACCC R: GACACAGAGATGTAGAAGGAG	2	220-272	4	6	0.22	0.23	1	--	--
	GT015	g, h	2	F: NED-GAGAATGGCTGGGCTCAGATC R: TTCCCTATTAGAGGCTCACGA	2	119-177	5	20	0.82	0.93	11	0.43	0.39
	415-416	b, g	2	F: HEX-GTTCCTTTCCTTACA R: ATCAATGTTTGTCAA	3	212-228	4	5	0.52	0.47	4	0.38	0.44
	EV104	c	2	F: HEX-TGGAGATGACAGGATTTGGG R: GGAATTTTTATTGTAATGGGTCC	4	143-163	8	10	0.52	0.81	6	0.61	0.62

Reference (Ref.): a, Kirkpatrick, 1992; b, Andersen et al., 1997; c, Valsecchi & Amos, 1996; d, Rosel et al., 1999; e, Bérubé et al., 1998; f, Palsbøll et al., 1997; g, Amos et al., 1993; h, Andersen et al., 2001. PCR, reaction in which the locus is amplified. The size range is derived from the MegaBACE-1000 DNA analyser. The size shift is the difference in allelic size observed between the two instruments used. A = number of alleles. H_O and H_e = observed and expected heterozygosity.

Engels, 1993), and the absence of overlap in allelic size range of markers from the same set.

Multiplex PCR Setting and Conditions

Each marker initially was screened for amplification separately. PCR products were separated and detected on 3% agarose gel. The forward primer of each locus was then 5'-end labelled with a fluorescent dye (FAM, HEX, or NED), which was chosen to analyse loci with similar allelic size but different fluorescent dyes simultaneously and to avoid size overlap among loci with the same dye. Based on size limitations and amplification specificity, two sets of microsatellite loci were made (*PPH1* and *PPH2*; Table 1).

Amplification of *PPH1* and *PPH2* was performed on a Mastercycler ep-gradient-S (Eppendorf) using the Multiplex PCR™ Amplification Kit (Qiagen), following the manufacturer's instructions. The Multiplex PCRs were conducted in a 10 µl reaction volume containing the Qiagen Multiplex PCR Master Mix (1X) (which included a "hotstart" *Taq*, dNTPs, and 3 mM of $MgCl_2$ as the final concentration), an equal concentration of 0.2 µM for each primer, about 50 ng of genomic DNA, and RNase-free water. Multiplex PCR conditions started with an initial activation step at 95° C for 15 min, followed by 35 cycles of denaturation at 94° C for 30 s, annealing at 52° C and 60° C for 90 s, respectively, for *PPH1* and *PPH2*, and an extension at 72° C for 60 s (30 min for final extension).

Because two loci of the *PPH2* set, 415-416 and EV104, had very different amplification conditions, they were amplified apart from the three other loci of the multiplex PCR and mixed together before loading on the DNA analyser. Amplification of the loci 415-416 and EV104 were conducted in a single PCR of 25 µl reaction volume using Qiagen and Promega *Taq* DNA polymerase, respectively. The PCR reaction mixture contained about 50 ng of genomic DNA, 0.8 U *Taq* DNA polymerase, thermophilic DNA polymerase Buffer (1X), 1.5 mM $MgCl_2$, 0.4 µM of primers, and 100 µM of dNTPs. The PCR reaction for the locus 415-416 started with an initial denaturation step at 95° C for 2 min, followed by a *Touchdown* protocol, with denaturation at 95° C for 1 min, annealing phase of 1 min starting at 50° C and decreasing by 1° C every cycle to 41° C followed by 35 cycles at 40° C, and extension at 72° C for 45 s (30 min for final extension). Regarding EV104, a PCR amplification in two steps was applied as described by Valsecchi & Amos (1996), except that the annealing temperatures were 50° C for the first annealing step and 56° C for the second. The PCR products of the two single PCRs were pooled with the multiplexed PCR No. 2 (Table 1) with a ratio of 7:7:2 µl, respectively, plus 2 µl of MilliQ water.

To check for repeatability of results among DNA analysers, two different instruments were used to detect each set of microsatellite loci (*PPH1* and *PPH2*): (1) a monocapillary ABI Prism 310 Genetic Analyser (Applied Biosystems) and (2) a 96-capillary MegaBACE-1000 DNA Analyser (Amersham Biosciences). Regarding the ABI PRISM 310 Genetic Analyser (Applied Biosystems), the PCR products of the two sets (*PPH1* and *PPH2*) were each diluted 20-fold, and 3 µl of these two solutions were each mixed with 16.5 µl ultragrade formamide and 0.5 µl Genscan 400-Rox size standard (Applied Biosystems). For the MegaBACE-1000 DNA Analyser (Amersham Biosciences), the PCR products were diluted for *PPH1* 5-fold and left as is for *PPH2*. From these two solutions, 3 µl were mixed with 6.7 µl of MilliQ water and 0.3 µl of MegaBACE ET400-R size standard (Amersham Biosciences).

Statistical Analysis

Genetic diversity of the samples from the Belgian coast and the Black Sea was characterised by the observed (H_o) and expected (H_e) heterozygosity and the number of alleles per locus (A) (Nei, 1987). We conducted exact tests to assess deviations from the Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium. These analyses were performed using *GENEPOP*, *Version 3.4* (Raymond & Rousset, 1995).

Results

The 11 microsatellite loci amplified successfully in four PCRs and were analysed in two electrophoresis runs: the first set (*PPH1*) contained six loci which co-amplified at once, and the second set (*PPH2*) required three PCRs to amplify five loci. The two DNA analysers provided good quality results for each microsatellite set and were fully consistent with each other. The comparison of allelic sizes between analysers (Table 1) indicated a constant locus-specific shift that ranged from one to eight base pairs (bp).

The mean number of alleles per locus was 11.0 in porpoises from the Belgian coast and 6.5 in those from the Black Sea, with one locus (GATA053) fixed in this area (Table 1). The mean observed/expected heterozygosity were 0.73/0.76 in Belgian porpoises and 0.51/0.49 in Black Sea porpoises (Table 1). There were no significant deviations in the observed genotype frequencies from the HWE at all loci, and no pair of loci yielded a significant linkage disequilibrium test after applying a Bonferroni correction (Rice, 1989).

Discussion

The number of alleles at each locus and the heterozygosity values (Table 1) observed in the harbour porpoises from the Belgian coast were in the same range of values as those reported in previous studies (Rosel et al., 1999; Andersen et al., 2001) and were much lower in the Black Sea population.

When comparing results from different analysers and/or laboratories, a constant shift in microsatellite allelic sizes is generally expected. In the present study, the comparison of two DNA analysers showed a constant locus-specific shift in size (Table 1). This shift is likely related to differences in the analyser's reagents, such as the size standards and/or the electrophoresis polymer, and it can also occur when different fluorescent dyes are used. Nevertheless, given the constancy of this shift, datasets from different laboratories or instruments can easily be corrected by calibration of allelic sizes. This calibration just requires the comparison of genotypes from reference samples shared between the different laboratories to determine the value of the shift and to apply the correction to the imported datasets.

These multiplex PCR systems will be a useful tool for rapid characterisation of harbour porpoise populations. They provide efficient polymorphic genetic markers and save expensive reagents, DNA samples, time, and work. As wide-scale study is increasingly needed to better understand the population structure worldwide, and thus their conservation status, these multiplex sets can be used as standard genetic tools for local study and provide the opportunity to combine calibrated datasets between laboratories for further large-scale meta-analyses.

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