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Isolation and characterisation of 26 microsatellite loci from a widespread tropical hydrozoan, *Macrorhynchia phoenicea* (Leptothecata, Aglaopheniidae), and cross-amplification in closely related species

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A B S T R A C T

We isolated and characterized 26 microsatellite loci for *Macrorhynchia phoenicea* (Busk, 1852), a rather common tropical hydrozoan from the Indo-Pacific. The number of alleles per locus ranged from 4 to 24. The observed heterozygosity ranged from 0.000 to 0.970 and the expected heterozygosity from 0.029 to 0.833. Ten loci were at Hardy–Weinberg equilibrium. No pair of loci presented linkage disequilibrium. Transferability of up to 18 loci was positive across four other Aglaopheniidae species from different genera. These loci will be used in studying reef population connectivity for these particular species at the scale of the Indo-Pacific, a promising but little explored research field.

1. Introduction

Hydrozoans are one of the most diverse marine suspension feeders, characterized by a wide range of reproductive modes and life cycles (Bouillon et al., 2006). While it is known that life history traits may affect population connectivity levels and diversification patterns (Schroth et al., 2002), this aspect has rarely been explored in hydroids, so that population genetics tools and relative data remain scarce for these organisms. To our knowledge, the only study of population genetics of hydrozoans using microsatellites focused on a freshwater invasive species in the Great Lakes region (USA) (Darling and Folino-Rorem, 2009).

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The Aglaopheniidae is one of the most diverse family of feather-like hydroids, comprising more than 250 valid species, some of those presenting wide geographical distribution ranges, from temperate to tropical seas or from the surface to several hundreds of meters depth (Bouillon et al., 2006). Useful information on evolutionary processes and dispersal capacities of marine species can be generated using co-dominant neutral molecular markers such as microsatellites (Donald et al., 2011). However, while well represented and largely distributed on coral reefs, microsatellite markers in Aglaopheniidae have only been developed, to date, on a single one (*Lytocarpia brevirostris* (Busk, 1852); Postaire et al., 2015).

Here we describe the isolation of 26 microsatellite markers for *Macrorhynchia phoenicea* (Busk, 1852), a common tropical hydroid of this family, measuring several centimeters, inhabiting tropical coral reefs from South Pacific islands to Africa, from 2 to 70 m depth (Millard, 1975). Furthermore, we tested these new markers on four phylogenetically closely related species: *Macrorhynchia philippina* (Kirchenpauer, 1872), *Macrorhynchia sibogae* (Billard, 1913), *Macrorhynchia spectabilis* (Allman, 1883) and *Lytocarpia nigra* (Nutting, 1905). Studying the genetic diversity of these species and their populations' structure is crucial for assessing coral reefs biodiversity and conservation planning, as little is know on the extent of cryptic speciation and levels of endemism among these organisms.

2. Material and methods

2.1. Construction of a microsatellite-enriched library and primer selection

Total genomic DNA of nine individuals (whole colonies cleared from reproductive structures) from Reunion Island was isolated using DNeasy Blood & Tissue kit (Qiagen™) following manufacturer's protocol and sent to GenoScreen, Lille, France (www.genoscreen.fr). One μg was used for the development of microsatellites libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. (2011). A total of 18,530 reads were obtained, of which 2958 contained microsatellite motifs. Obtained sequences were analyzed with QDD (Meglécz et al., 2010). Among the 204 primer pairs designed to amplify fragments containing a microsatellite motif, and on the basis on our criteria (PCR product > 100 bp and number of repeats ≥ 6), we selected 96 pairs of primers and tested their polymorphism on 16 individuals (eight from Reunion Island and eight from New Caledonia). Forward primers were indirectly fluorochrome labeled (6-FAM) by adding a 19-bp M13 tail at their 5′ end (5′- CACGACGTTGTAAAACGAC-3′) (Schuelke, 2000). Each amplification reaction was performed in a total volume of 10 μL: 5 μL of MasterMix Applied 2x (Applied Biosystems), 0.25 μL (1 μM) of forward primer tagged with the M13 tail (final concentration: 0.025 μM), 0.25 μL (10 μM) of reverse primer (final concentration: 0.25 μM), 0.25 μL (10 μM) of fluorescent dyed M13 tail (final concentration: 0.25 μM) and 2 μL (10 ng/μL) of genomic DNA (final concentration: 2 ng/μL). The thermocycling program was as follows: 94 °C for 5 min + 7 x (94 °C for 30 s, 62 °C [−1 °C at each cycle] for 30 s, 72 °C for 30 s) + 30 x (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) + 8 x (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) for the final hybridization of fluorescent dyed M13 tail + 72 °C for 5 min. PCR products were genotyped using an ABI 3130 genetic analyzer (Applied Biosystems) and allelic sizes were determined using GeneMapper v4.0 (Applied Biosystems). Primer pairs were eliminated from further development when (1) they failed to amplify, (2) they amplified multiple fragments, or (3) genotype scoring was deemed unreliable. Finally, a total of 26 candidate loci were successfully kept for characterizing polymorphism on a single population.

2.2. Polymorphism testing and microsatellite cross-amplification

Genotyping of these 26 markers was conducted on 34 individuals from a *M. phoenicea* population from Reunion Island (Saint Pierre; 21.35015 S, 55.48202 E) and, to explore allelic richness at large geographic scale, on individuals from Juan de Nova Island (Scattered Islands; n = 16), Madagascar (n = 16) and New Caledonia (n = 16) using the same protocol as above. A different dye was assigned to each locus (6-FAM, VIC, NED, PET) and PCR products were pooled in panels according to amplified fragment sizes and dyes to maximize efficiency and minimize costs. Cross-amplifications of these 26 markers were also tested using the same protocol on *M. philippina*, *M. sibogae*, *M. spectabilis* and *L. nigra*.

2.3. Data analysis

Allele sizes and number of alleles per locus are indicated for the whole dataset (i.e. all sampling sites from South Western Indian Ocean and Pacific); population statistics were calculated for the Reunion Island population only (n = 34). Diversity indices were assessed and conformation to Hardy−Weinberg expectations tested using Arlequin v 3.5.1.2 (Excoffier et al., 2005). Tests of linkage disequilibrium between all pairs of loci were conducted using Genepop v 4.2. Null alleles and other potential technical biases were tested using Micro-Checker v2.2.3 (van Oosterhout et al., 2004).

3. Results and discussion

All 26 loci were polymorphic. From the genotyping of the Indo-Pacific sampling, the number of alleles per locus ranged from 4 to 24; in the tested population from Reunion Island, the number of alleles ranged from 2 to 7. Observed heterozygosities in the Reunion Island population ranged from 0.000 to 0.970 and expected heterozygosities from 0.029 to 0.833 (Table 1). Over 325 linkage disequilibrium tests between all pairs of loci, only 16 were significant after False Discovery Rate

Table 1
Microsatellite loci developed for *Macrorhynchia phoenicea* and their primer sequences (F: forward, R: reverse). Accession numbers in GenBank are indicated. Diversity indices are issued from Arlequin v3.5.1.2. N_a: number of alleles per locus at the Indo-Pacific scale; $N_a pop$: number of alleles per locus in the tested population (Reunion Island); N: number of genotyped individuals; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient. Under the F_{IS} are indicated the significance of the P-values for deviation to Hardy–Weinberg equilibrium (1000 permutations): NS: non significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Null allele frequencies (r) are issued from Micro-Checker v 2.2.3 (van Oosterhout et al., 2004), and presence or absence is indicated below (yes or no).

Locus name	Primer sequence (5′–3′)	GenBank accession number	Repeat motif	Dye	Panel	Size (bp)	N_a	$N_a pop$	N	H_O	H_E	F_{IS}	r
Mp01	F: GGCAACTAACAACCGCATTT R: AATAAGTCAGAAGCATTATGGTCAA	KM675787	$(TATC)_{13}$	VIC	1	99–170	16	7	34	0.206	0.833	0.756 ***	0.360 yes
Mp02	F: CCAATAACATGCATGCAACA R: TCGAACCTTGTGTCCTTGTG	KM675788	$(CAA)_9$	6-FAM	1	106–133	8	2	34	0.029	0.029	0.000 NS	−0.014 no
Mp03	F: GGTTCAAATTAAAAGACGTCCC R: TCCTAACCCTTTTCTTACCCC	KM675789	$(ACA)_7$	NED	1	153–194	13	2	34	0.029	0.275	0.895 ***	0.291 yes
Mp04	F: CTTCCAGCCATGAAAGTTGA R: ATACGCTCCTGGATTGGTTG	KM675790	$(CAA)_7$	PET	1	150–189	14	2	34	0.000	0.506	1.000 ***	0.414 yes
Mp05	F: TGCTAATTATAGACAGGTTACATTTGA R: TTGGGCTAGGTAACATTACCACT	KM675791	$(TAGC)_5$	6-FAM	1	228–256	6	3	34	0.970	0.534	−0.840 ***	−0.733 no
Mp06	F: CGAGAATTCCACCCAATG R: CGACGATTTACTACAAGTCCAAGA	KM675792	$(TGT)_{11}$	VIC	1	268–333	17	4	34	0.029	0.244	0.881 ***	0.268 yes
Mp07	F: ATGCGTATGCAAAGGATTGG R: AAGGAGGCAGTTGCTATGGT	KM675793	$(GA)_9$	6-FAM	2	140–164	10	3	34	0.029	0.086	0.663 NS	0.142 yes
Mp08	F: GTCAAGTTGGTTTCACTCATGC R: ACAAGCAACGATGAAGGGAC	KM675794	$(GTT)_7$	VIC	2	155–167	10	3	34	0.323	0.586	0.452 ***	0.231 yes
Mp09	F: ATTGATTCACGCTTGTGCAG R: GGTTGTTAGATCAGGTAATGTGG	KM675795	$(AG)_7$	NED	2	176–196	10	2	34	0.059	0.058	−0.015 NS	−0.030 no
Mp10	F: TTATCCCGCTAAACATGAAACA R: CGCGTTATGTAGATCAGCCA	KM675796	$(CGTTGT)_5$	6-FAM	2	236–347	24	3	34	0.000	0.262	1.000 ***	0.308 yes
Mp11	F: GGAAAACGACCACAATCCAA R: CTCTTGATTTGATTCAGGAATGT	KM675797	$(CTAT)_9$	VIC	2	289–338	10	3	34	0.235	0.643	0.638 ***	0.288 yes
Mp12	F: CGAGTCGTTGCAGGGTTACT R: TCGACGTTATATCTCAAACCTCA	KM675798	$(AGAT)_7$	VIC	3	135–223	21	3	34	0.088	0.319	0.727 ***	0.260 yes
Mp13	F: CTTCACCGCTATTAGCTCTTCA R: TTCATAGCGATACGAAACACG	KM675799	$(GA)_7$	6-FAM	3	138–164	8	2	34	0.000	0.395	1.000 ***	0.372 yes
Mp14	F: TTTCTCCAAATTGTTCCAAGC R: GCAAATGCCTAAGCTGCAA	KM675800	$(GTT)_6$	NED	3	177–204	7	2	34	0.000	0.058	1.000 NS	0.157 yes
Mp15	F: GAGGCGTTTCCTTTTCAAAT R: GAACGTTTCCTCGGAGTGAC	KM675801	$(CAA)_9$	PET	3	202–228	9	3	34	0.176	0.388	0.548 ***	0.228 yes
Mp16	F: TCAGAGTTCAAGGGCCAAGT R: TGGGAAGTGTGACCTCGATT	KM675802	$(AAC)_6$	6-FAM	3	197–218	6	2	34	0.029	0.187	0.845 ***	0.236 yes
Mp17	F: GCTTTCTCAACAGTTTTCTATTGG R: CGTGTGAGCCTTTCACTTGAT	KM675803	$(TG)_9$	6-FAM	4	114–144	15	6	34	0.618	0.798	0.228 ***	0.096 yes
Mp18	F: TCCCACTGCGTTGTATTGTG R: GGAAATAATTGGTTTCCAGACAA	KM675804	$(ATCT)_6$	VIC	4	127–180	16	6	34	0.588	0.727	0.194 NS	0.077 no
Mp19	F: CAGACATAAATTACGAATAATTAACCA R: CCTGCTTTTCTCTTTTGCTTTC	KM675805	$(GT)_9$	NED	4	153–174	5	3	34	0.412	0.505	0.218 NS	0.097 no
Mp20	F: CGCTGACCAGTCCGTTTAAT R: GTTGAAGTTCACCAACGGCT	KM675806	$(GTT)_6$	PET	4	192–204	5	2	34	0.029	0.029	0.000 NS	−0.015 no
Mp21	F: TCCGTTGTGTCTATGATATGTTCTT R: CCACATGGTTATCGTCAGCA	KM675807	$(TTG)_7$	6-FAM	4	271–315	16	4	34	0.265	0.331	0.204 NS	0.072 no
Mp22	F: TTCGCTCTTTCATTCATCCG R: GAGAAAAGAAATGAAACGCACA	KM675808	$(TG)_6$	6-FAM	5	119–129	6	4	34	0.176	0.518	0.663 ***	0.287 yes
Mp23	F: GGCGACGAAGATTTGAGAAC R: GAGTGTGGATGTTGCGTGAG	KM675809	$(AAC)_{12}$	VIC	5	126–168	15	4	34	0.353	0.347	−0.017 NS	−0.036 no
Mp24	F: AATACGGCGTCAGAAACCTC R: GCGATCCACCAAATGCTTAT	KM675810	$(CT)_8$	NED	5	136–164	24	4	34	0.118	0.528	0.780 ***	0.331 yes
Mp25	F: CACTGGACTACGATCACTCATTG R: AGGAGACGAGAATGTCGGAA	KM675811	$(TGT)_7$	PET	5	157–169	4	3	34	0.059	0.437	0.867 ***	0.340 yes
Mp26	F: CAAGATCAAGGACACCGAGC R: TCAAATCATCGCCACTAGACA	KM675812	$(CAA)_6$	6-FAM	5	219–242	9	2	34	0.000	0.058	1.000 NS	0.157 yes

(FDR) correction for multiple testing (Benjamini and Hochberg, 1995). In the tested population, 10 loci over 26 were at Hardy–Weinberg equilibrium. All the other loci showed significant heterozygotes deficit (F_{IS} ranging from 0.228 to 1) except Mp05 ($F_{IS} = -0.84$). On one hand, this is potentially due to the presence of null alleles, revealed using Micro-Checker v2.2.3 (van Oosterhout et al., 2004, Table 1). However, no null homozygotes were found, indicating that if null alleles are present, they may occur in low frequencies. Significant heterozygotes deficit, on the other hand, is consistent with mating between related individuals for these loci and might reflect the specific sexuality (gonochoric) and mode of larval development of this species. Indeed, like the majority of Aglaopheniidae, *M. phoenicea* is a brooder species, incubating its larvae in dedicated

Table 2
Cross-amplification for 26 microsatellite markers designed for *Macrorhynchia phoenicea* across four Aglaopheniidae species: *Lytocarpia nigra* (n = 6), *M. philippina* (n = 10), *M. sibogae* (n = 1) and *M. spectabilis* (n = 1) (+, amplified; +P, polymorphic; -, no amplification). Size ranges of the PCR product in base pairs (19 bp M13 tail included) and number of alleles (in brackets) are also indicated.

Locus Name	*L. nigra* (n = 6)	*M. philippina* (n = 10)	*M. sibogae* (n = 1)	*M. spectabilis* (n = 1)
Mp01	–	–	–	–
Mp02	–	–	–	–
Mp03	+P 162–165 (2)	+P 162–165 (2)	–	+P 165–168 (2)
Mp04	+162 (1)	+P 159–162 (2)	–	+P 162–174 (2)
Mp05	+P 230–256 (3)	+P 230–256 (4)	–	+P 232–240 (2)
Mp06	+P 305–311 (3)	+P 292–311 (3)	–	+P 305–311 (2)
Mp07	+140 (1)	+P 144–150 (4)	–	+148 (1)
Mp08	+P 159–167 (4)	+P 155–167 (5)	–	+P 161–165 (2)
Mp09	+P 186–188 (2)	+P 186–188 (2)	+188 (1)	+186 (1)
Mp10	+253 (1)	+P 253–269 (4)	–	+253 (1)
Mp11	–	–	–	–
Mp12	–	–	–	–
Mp13	+P 154–160 (2)	+160 (1)	–	+162 (1)
Mp14	+182 (1)	+182 (1)	–	+182 (1)
Mp15	+P 210–225 (4)	+P 213–225 (3)	–	+P 208–216 (2)
Mp16	+212 (1)	+P 212–215 (2)	–	+212 (1)
Mp17	+P 130–134 (2)	+P 124–134 (4)	–	+P 128–130 (2)
Mp18	+144 (1)	–	–	–
Mp19	–	+159 (1)	–	+P 164–178 (2)
Mp20	+P 198–201 (2)	+P 198–201 (2)	+P 198–201 (2)	+201 (1)
Mp21	–	+291 (1)	–	+P 272–275 (2)
Mp22	+P 121–129 (3)	+P 121–127 (3)	+P 121–127 (2)	+121 (1)
Mp23	+P 136–139 (2)	+P 136–139 (2)	+P 136–139 (2)	+P 132–142 (2)
Mp24	+143 (1)	+P 141–145 (3)	–	+P 154–156 (2)
Mp25	–	–	–	+169 (1)
Mp26	+P 222–233 (4)	+P 227–236 (2)	+236 (1)	+233 (1)

structures. When mature, released planulae usually crawl (rather than swim) for a short distance until metamorphosis, which usually starts within less than a day (Sommer, 1990). The only true planktonic phase of their life cycle corresponds to the release of spermatozoids in the water column, which life span has been studied for only one other hydroid species, and seems to be reduced to a few hours (Yund, 1990). In addition, Aglaopheniidae species do not seem to reproduce during mass spawning events like many scleractinians: in several species in Reunion Island coral reefs, the presence of reproductive structures have been observed during several months (CAFB pers. obs.). In temperate marine hydrozoans species, the reproduction period is related to environmental factors and consequently colonies in the same location tend to reproduce at the same time (Gili and Hughes, 1995). Thus fertilization occurs preferentially between geographically close individuals, which may be potentially related. All these aspects naturally contribute to low levels of genetic diversity within populations, as migration events are potentially rare. The assumption of reduced gene flow among populations will be further addressed in a population genetic study of this species with a broad geographical coverage, from South-Western Indian Ocean to Pacific.

Cross-species amplification tests revealed that various loci amplified across closely related species of this family (Table 2). Among the 26 loci, (1) 12 and 9 were polymorphic while amplifying *M. philippina* (n = 10) and *L. nigra* (n = 6), respectively; (2) *M. spectabilis* (n = 1) and *M. sibogae* (n = 1) were heterozygotes for 11 and 3 loci, respectively. This shows high transferability within the same genus, and between genera from the same family. These loci might be useful for other species within this family that have not been tested in this study. More generally, these loci will be useful in phylogeographic studies, potentially revealing biogeographic barriers, and will help to investigate relationships between larval dispersal capacities and gene flow of such coastal benthic species.

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