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SHORT COMMUNICATION

Highly polymorphic microsatellite loci for the Acapulco damselfish, *Stegastes acapulcoensis*, and cross amplification in three congeneric species

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Abstract In the present study we report a set of 13 novel microsatellites isolated from and characterized for the Acapulco damselfish *Stegastes acapulcoensis*, a species endemic to, but widely distributed within, the Eastern Tropical Pacific (ETP) region. The loci were tested in 30 individuals from the Colombian Pacific and were highly polymorphic. The mean allele number per locus was 19.5 (± 4.03 SD) and the observed heterozygosities ranged from 0.192 to 1. Some of the loci were successfully cross-amplified and were polymorphic in three *Stegastes* species from the ETP: *S. arcifrons*, *S. flavilatus* and *S. beebei* (with 11, three and seven amplified loci, respectively). The high variability and cross-amplification success of the new set of microsatellites reported here allows these markers to be a useful resource for genetic studies of *S. acapulcoensis* and some of their congeners to address evolutionary, ecological and conservation-related questions in the Eastern Tropical Pacific.

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Introduction

Damselfishes of the genus *Stegastes* inhabiting the Eastern Tropical Pacific (ETP) comprise a group of eight species, all endemic to this region and characterized by contrasting patterns of geographic distribution. Some species are oceanic island endemics with remarkably restricted ranges, inhabiting the small and isolated islands in the region, while others have some of the widest distributions on the continental coast in the ETP (Robertson and Allen 2015). The vast differences in geographic range size of these closely related reef fishes and the overlapping distribution and coexistence of some of them make this group of species an ideal model to conduct studies addressing a variety of evolutionary and ecological questions (Ruttenberg and Lester 2015). The Acapulco damselfish, *S. acapulcoensis,* is one of the most widely distributed species of this group (Robertson and Allen 2015). This species is common along shallow rocky reefs and areas with coral growth. Adults are strongly territorial and defend algal mats that they grow within their territory (Wellington 1982).

Like many marine organisms, *S. acapulcoensis* has the potential to disperse during the pelagic larval phase and to colonize new habitats beyond its native geographic range. This damselfish occurs mainly along the continental coast, from the central Gulf of California to Chile (Robertson and Allen 2015). Despite the distances separating the continental coast from the offshore islands (between 380 and 1075 km), some vagrants are transported across long distances and reach the islands (Rodríguez-Moreno et al. 2011; Robertson and Allen 2015). Although resident populations were thought

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to be absent on offshore islands, there is evidence of a possible recent colonization of the Galápagos Islands by *S. acapulcoensis* (Victor et al. 2001). This has been evident from an incremental increase of recruits following the 1982–83 El Niño–Southern Oscillation (ENSO) event (Grove 1984); the continued arrival of recruits (Victor et al. 2001) and the persistence of adults with a range of age classes (Meekan et al. 2001). At Malpelo Island, on the other hand, most observed individuals have been juveniles (Rodríguez-Moreno et al. 2011). The wide geographic distribution and colonization potential of *S. acapulcoensis* makes this species an interesting model to study patterns of dispersal, population structure, demographic events and possible colonization routes between the continent and offshore islands in the ETP.

Microsatellites have been recognized as useful and reliable genetic markers to study a wide range of evolutionary and ecological processes (Selkoe and Toonen 2006). Some features that characterize these markers as widely applicable are high levels of allele polymorphism, codominant inheritance and high reproducibility (Chistiakov et al. 2006). The high resolution of genetic information obtained from microsatellites can be especially useful to estimate contemporary larval dispersal, levels of genetic diversity, population structure, migration rates, and demographic events among other key population processes (Selkoe and Toonen 2006).

The present study aimed to develop a set of polymorphic microsatellites specific for the Acapulco damselfish, *S. acapulcoensis,* useful to study fine-scale population structure and patterns of dispersal and connectivity of this model species. Additionally, cross-amplification success in congeneric species from the ETP was tested to characterize their utility in other ETP *Stegastes* species. Currently, no microsatellite markers specifically developed for *S. acapulcoensis* or for any other *Stegastes* in the Eastern Pacific exist. Nevertheless, two sets of microsatellites have been previously published for *S. partitus*, a congeneric species from the Caribbean (Williams et al. 2003; Thiessen and Heath 2007). Some of these markers successfully cross-amplified in some ETP *Stegastes* species (M. Rodríguez-Moreno, unpublished data) and were polymorphic (Urbiola-Rangel and Chassin-Noria 2013), but their suitability for genetic studies needs to be further investigated. Here, we isolated and characterized a set of 13 novel microsatellites specific for *S. acapulcoensis* and tested cross-species amplification on *S. arcifrons, S. flavilatus* and *S. beebei* from the ETP to enable population genetic studies in this group of species.

Methods

Genomic DNA was extracted from fin clips and muscle tissue preserved in 96% ethanol. Two different methods were used for DNA extraction: either a salting out extraction, described by Sunnucks and Hales (1996), with a few modifications or a DNeasy Blood and Tissue Kit (Qiagen), according to manufacturer protocols. Extracted DNA was stored at −20 °C and dilutions were made as required for use in PCR at 50 ng/ul. Microsatellite loci were isolated and developed as follows: genomic DNA from a single individual of *S. acapulcoensis* (collected from Negritos, Bahía Málaga) was used to create an enriched microsatellite library, by using five tetranucleotide repeat motifs [$(AGAT)_8$, $(AAAT)_8$, $(ACAT)_8$, $(AAGT)_8$, $(AACT)_8$] to identify clones containing these motifs, following the SuperSNX-streptavidin bead enrichment protocol of Glenn and Schable (2005).

Primer pairs for 23 isolated microsatellite loci were developed and initially tested for amplification and polymorphism on five to 15 specimens of *S. acapulcoensis* collected from three different locations in the Colombian Pacific (Bahía Málaga, Gorgona Island and Cabo Marzo). Primers were tested on single locus PCRs at annealing temperatures ranging from 42 to 62 °C. PCR reactions were conducted in a total volume of 10 μl containing 20–100 ng DNA, 1X PCR buffer, 0.6 mM dNTP mix, 2.5–3.5 mM $MgCl_2$, 1 $mg.ml^{-1}$ BSA, 0.16 μM of fluorescently labeled universal M13 primers (FAM, VIC, NED and PET), 0.04 μM of unlabeled forward primer, 0.16 μM of unlabeled reverse primers (Schuelke 2000) and 1 U Taq Polymerase. Thermal cycling conditions proceeded as follows: An initial denaturation of 94 °C for 4 min, followed by 30 cycles of 94 °C for 15 s, annealing temperature gradient between 42 and 62 °C for 15 s, extension at 72 °C for 45 s, followed by eight cycles at 94 °C for 15 s, 53 °C for 15 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min.

Thirteen of the 23 assayed loci amplified successfully in the focal species, *S. acapulcoensis,* and were polymorphic. The 5′ ends of the Forward primers of the 13 selected loci were then labeled with one of the following fluorescent dyes: 6-FAM, NED, VIC and PET (Life Technologies) and combined into four multiplex mixes (as per Table 1). Selected loci were tested by genotyping DNA from 30 specimens of *S. acapulcoensis* from a single population in the Colombian Pacific (Los Negritos, Bahía Málaga). Cross-amplification of these loci was also tested in three other *Stegastes* species from the Eastern Pacific: *S. arcifrons* ($n = 30$), *S. flavilatus* ($n = 3$ to 20) and *S. beebei* ($n = 5$). PCR-amplification of all loci (except for *S. flavilatus*) was performed on a Bio-Rad C1000 Thermocycler with a touch-down procedure with the following cycling conditions: a 5 min initial denaturation step at 95 °C; 10 cycles of 30 s at 95 °C, 90 s at Multiplex Primer Mix specific temperature (see Table 1) and 30 s at 72 °C, followed by 18 cycles using the same conditions and a reduction of 3 °C in annealing temperature. The final elongation step was performed at 60 °C for 30 s. Each 10 μl multiplex PCR amplification reaction contained 50 ng DNA, 5 μl (2×) Type-It Multiplex Master Mix (Qiagen) and 1 μl Multiplex

Table 1 Characterization of 13 microsatellites loci isolated for *Stegastes acapulcoensis*. The table shows size range of genotyped fragments, sample size (N), number of alleles (N_a), fluorescent dye label (Dye) and multiplex primer mix in parentheses, observed (H_o) and expected (H_e) heterozygosity, polymorphism information content (PIC), Hardy–Weinberg equilibrium probability (pHWE) and null allele frequency (F_{NA}). Significant *p*-values, after correction for multiple testing using Bonferroni correction (Benjamini and Hochberg 1995), are in bold (significance level $p < 0.05$)

Locus	Repeat Motif	Primer Sequence (5′-3′)	Size (bp) in original clone	Size range (bp)	N	N_a	Dye (Multiplex)	H_o	H_e	PIC	pHWE	F_{NA}	GeneBank Accession Number
Stac 09	$(GATA)_{25}$	F: ACACAAATAACTGAATCAATACA	271	182–282	30	20	6-FAM (A)	0.967	0.941	0.920	0.501	-	KY249581
		R: GGCCTAGGGTCTGCAAACAT											
Stac 122	$(AGAT)_{17}$	F: GCTAACTTCAGCAAAGGACCT	326	184–340	26	23	VIC (A)	0.423	0.951	0.929	**0.000**	0.2730	KY249582
		R: ACAGATGCTCATATGAAAGTCA											
Stac 111	$(AGAT)_{17}$	F: GCAGTGTGCAAAATGACTGGT	212	120–216	30	17	PET (A)	0.900	0.916	0.893	0.642	-	KY249583
		R: ACGAACATATAAAGCAGTCTCAGAGT											
Stac 134	$(CTTT)_{16}$	F: AGACTGTTGAAATAAACAAAAACT	193	128–188	29	15	NED (A)	0.724	0.922	0.898	**0.005**	0.1010	KY249584
		R: CCTTCAACCGCTGCATTTCC											
Stac 10	$(TCTA)_{20}$	F: CAACCAAATTTCCCCTTGGGG	280	203–271	30	16	6-FAM (B)	0.867	0.936	0.914	0.103	-	KY249585
		R: TTTGTTTTGCATGCAGGCCA											
Stac 159	$(ATAG)_{24}$	F: GGGACTTCTCAGCTGAGCAG	207	130–322	27	21	VIC (B)	0.481	0.944	0.922	**0.000**	0.2385	KY249586
		R: GGCACTATAATCTTGGACAAGGG											
Stac 79	$(GATA)_{14}$	F: AGCGACAACTCAATGCATGA	213	163–231	30	21	PET (B)	0.900	0.941	0.920	0.6933	-	KY249587
		R: TCCTTTGGATATAACACACTGT											
Stac 125	$(ATCT)_{23}$	F: CTTTGGCACTGAACCCCTCA	389	298–386	30	18	6-FAM (C)	0.933	0.925	0.899	0.708	-	KY249588
		R: TCCACAAACAATATCCACCCAGT											
Stac 142	$(ATAG)_{25}$	F: ACAGGTGGCGATGCAAAAAC	281	214–306	30	21	NED (C)	0.800	0.945	0.925	0.071	0.0695	KY249589
		R: ACTGGTTTGCATATTTGACCGT											
Stac 155	$(TCTA)_6$ CCTA	F: ACAACCAAATTTCTCCTTGGGG	147	104–164	26	13	PET (C)	0.192	0.901	0.873	**0.000**	0.3822*	KY249590
	$(TCTA)_{10}$	R: CTGCTGATACCACTACCGGT											
Stac 163	$(GATA)_{25}$	F: AGCTCAACTGTTTAAGACTT	211	144–228	27	19	6-FAM (D)	0.704	0.944	0.922	**0.000**	0.1212	KY249591
		R: TAAACCCTGAATGCATGTT											
Stac 128	$(AGTT)_{11}$	F: TGCTGCATTTCTAGGAGGGT	217	148–240	30	21	NED (D)	1.000	0.947	0.927	0.761	-	KY249592
		R: CTGAAACTGCAGCCTCCTCT											
Stac 147	$(GATT)_{20}$	F: ACTCCCTTGTTTGATGCTCAG	334	222–398	30	29	PET (D)	0.867	0.967	0.949	0.089	0.0448	KY249593
		R: CACACCTCACTCTTCAGACT											

*A largely unscorable imperfect (interrupted) repeat locus in most samples screened

Annealing temperature for multiplex primer mix A: 60 °C; B: 53 °C; C: 57 °C and D: 50 °C

Primer Mix (2 uM each). Amplification was confirmed by 1.5% agarose gel electrophoresis. Cross-amplification for *S. flavilatus* was tested using single locus PCRs as described above. Fragment analysis of the initial single PCR tests and subsequent multiplex PCRs were run on an ABI3730 (Applied Biosystems) genetic sequencer with a 725 bp size standard (Alexa-725; Maddox and Feldheim 2014). Allele sizes were scored using Geneious 8.1.7 (Biomatters).

Scoring errors, allele dropout and presence of null alleles were checked using Micro-Checker 2.2.3 (Van Oosterhout et al. 2004). The software PGDSpider (Lischer and Excoffier 2012) was used for data format conversions for programs to be used subsequently. Allelic diversity, observed (H_o) and expected (H_e) heterozygosities of each locus were calculated using the program Arlequin (ver 3.5.2.2). Departure from Hardy-Weinberg Equilibrium (HWE) was evaluated with a probability test using the Markov chain method (10,000 dememorizations, 1000 batches and 10,000 iterations per batch), implemented in GENEPOP ver. 4.5.1 (Raymond and Rousset 1995; Rousset 2008). Null allele frequencies were calculated with Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) and the Polymorphic Information Content (PIC) of each locus were estimated using the program Cervus 3.0.7 (Kalinowski et al. 2007). In codominant markers, such as microsatellites, the PIC value ranges from 0 to 1, with higher values being more informative (Hildebrand et al. 1992). The null hypothesis for linkage disequilibrium was tested through pairwise comparisons of loci in FSTAT version 2.9.3.2 (Goudet 2002) based on 7800 permutations. The Bonferroni correction method (Benjamini and Hochberg 1995) was used to adjust significant levels for multiple testing (HWE and linkage disequilibrium).

Results and discussion

Of the 23 microsatellites isolated, 13 successfully amplified and were highly polymorphic in *S. acapulcoensis*. The allele number per locus ranged from 13 to 29 with a mean of 19.5 (± 4.03 SD; Table 1). Mean observed (H_o) and expected (H_e) heterozygosities were 0.75 (± 0.24 SD) and 0.94 (± 0.02 SD), respectively. Five loci out of 13 deviated from HWE after Bonferroni correction for multiple testing ($p < 0.05$). Homozygote excess related with the presence of null alleles or stochasticity due to the small sample size ($n = 30$) might explain these deviations. Results from Micro-Checker suggested the presence of null alleles for seven loci, five of them deviating from HWE. Null allele frequencies ranged from 10 to 38%, all remaining loci lacked evidence of null alleles. After Bonferroni correction, no linkage disequilibrium was detected between any locus pair. The polymorphic information content was high, ranging from 0.873 to 0.929. Markers with these levels of polymorphism are considered highly informative (Hildebrand et al. 1992). The large number of alleles found for these loci can improve the power of statistical analysis if the number of samples and the number of loci available are low (Selkoe and Toonen 2006). The developed microsatellites should be useful for a variety of genetic analyses (i.e., population structure, parentage assignment) and contribute to the genetic resources available for evolutionary,

Table 2 Cross-amplification results of 13 microsatellites developed for *Stegastes acapulcoensis* tested on three *Stegastes* species from the Eastern Tropical Pacific. Number of individuals tested in parenthesis, observed (H_o) and expected (H_e) heterozygosity (NA: no amplification of PCR products and NS: non- specific amplification)

	S. arcifrons (20)				*S. flavilatus* (20)				*S. beebei* (5)			
Locus	Size range	No. of alleles	H_o	H_e	Size range	No. of alleles	H_o	H_e	Size range	No. of alleles	H_o	H_e
Stac 10	167–303	16	0.800	0.872	232–300	12	0.750	0.849	219–251	6	0.800	0.911
Stac 79	162–234	15	0.950	0.940	162–422	21	0.750	0.950	166–234	7	0.600	0.911
Stac 09	202–326	13	0.900	0.918	226–278	13	0.700	0.905		NA		
Stac 111	120–188	11	0.650	0.706		Monomorphic			102–122	2	1	1
Stac 155	192–228	10	0.550	0.878		Monomorphic				Monomorphic		
Stac 122	160–224	14	0.550	0.919		NA[a]				Monomorphic		
Stac 142	190–270	11	0.631	0.890		NA[a]				Monomorphic		
Stac 159	154–210	15	1.000	0.942		NA[a]			230–258	2	1	1
Stac 125	310–358	11	0.900	0.914		NA[a]			306–378	8	1	0.956
Stac 128	160–164	2	0.250	0.224		NA[a]			160–176	5	0.600	0.800
Stac 147	306–414	19	0.900	0.937		NS[a]			338–434	7	1	0.911
Stac 134		NA				NA[a]				NA		
Stac 163		NA				NA[a]				NA		

[a] Cross amplification tested in three samples at least in two PCR reactions

ecological and conservation investigations in marine reef fishes.

Cross-amplification of the microsatellites was successful in *S. arcifrons* for 11 loci, in *S. flavilatus* for three loci and in *S. beebei* for seven loci (Table 2). The levels of polymorphism of the amplified loci were high in *S. arcifrons* with a mean of 13.5 (± 2.8 SD) alleles per locus (excluding locus Stac128) and in *S. flavilatus* with a mean 15.3 (± 4.9 SD) alleles per locus. Loci that amplified in *S. beebei* will need further testing to evaluate polymorphism as we tested the markers on only five individuals but, even with such a small sample, the mean number of alleles per locus was considerable (5.0 ± 2.4 SD). The differential amplification success rate across species evident here might reflect the phylogenetic relationships between species (Estoup and Angers 1998); however, phylogenetic relationships for this group of species have not yet been resolved. The set of microsatellites for congeners of *S. acapulcoensis* identified here can be expanded using previously published microsatellites for a congeneric species from the Caribbean (*S. partitus*). Although some of these published markers successfully cross-amplified in some *Stegastes* from the ETP (Rodríguez-Moreno, unpublished data) and four were polymorphic in *S. acapulcoensis* (Urbiola-Rangel and Chassin-Noria 2013), their suitability for genetic studies needs to be further investigated. The alternative cross-amplification approach to obtain useful markers without developing species specific markers seems to work across some *Stegastes* species, thus bypassing the costs and time associated with marker development.

The 13 microsatellite markers reported here represent the first set of loci developed for *S. acapulcoensis*. The high variability and cross-amplification success allows these markers to be applied in population genetic studies of this species and some of their congeners. These markers are currently being used to study the fine-scale population genetic structure and patterns of dispersal and connectivity of *S. acapulcoensis* and *S. arcifrons* in the Equatorial Eastern Pacific. This information is being used to assess the effectiveness of marine protected areas in terms of connectivity in the Colombian Pacific and the Eastern Tropical Pacific Marine Corridor-CMAR (http://www.cmarpacifico.org). Ultimately, these markers represent an effective tool to further track the possible origins of larvae arriving at localities outside their native geographic distribution and to unravel potential processes influencing the biogeographic patterns of reef fishes in the ETP.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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