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Genetic Diversity of the Endemic Xantus' Hummingbird Using 16 Novel Polymorphic Microsatellite Loci, and Their Cross Amplification between Six Related Species

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Abstract

A set of 16 microsatellite loci was developed and characterized for the Xantus' hummingbird (*Hylocharis xantusii*) by using 454 next-generation sequencing. Twenty-five *H. xantusii* samples from one population were genotyped; all loci were polymorphic, with the number of alleles ranging from three to ten. The mean observed heterozygosity was 0.681 for all loci. No significant linkage disequilibrium was detected, but five loci (Hxan05, 06, 09, 13 and 14) showed deviation from Hardy-Weinberg equilibrium. These microsatellite loci are the first to be characterized for *H. xantusii*. A moderate to high level of cross-species amplification was observed across the six hummingbird species (31% - 87.5%), with the best cross amplification results observed in the closest related species *(H. leucotis*, *Cynanthus latirostris*, *Calypte costae*). The availability of these molecular tools allows assessing questions integrating population genetics, ecology, conservation, and evolutionary history for *H. xantusii* and for other phyogenetically related species.

Keywords

Baja California Peninsula, *Hylocharis xantusii*, Microsatellites, Pyrosequencing, Cross-Species Amplification

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1. Introduction

Endemic species with a narrow distribution and specific habitat requirements are usually vulnerable to ecological disturbances and environmental changes (e.g. deforestation, introduced diseases, competition and predation pressures) [1]. Therefore, it is fundamental to develop long-term conservation plans of these kinds of species based on habitat and genetic information, such as genetic diversity [2]. Microsatellites are one of the most popular genetic markers for a wide range of applications in population genetics, conservation biology, and evolutionary biology. Loci developed using genomic sequencing for microsatellite markers design offer the opportunity to obtain enough genetic information for species where genetic data are limited [3].

The Xantus' hummingbird *Hylocharis xantusii* (Lawrence 1860) is endemic to central and southern Baja California Peninsula (BCP); limited information is available regarding its biology and ecology [4], but it has been observed that its distribution is discontinuous, having four genetic populations mainly associated to oases [5]. This species can be found in oak-pine and tropical deciduous forests, oases, canyons and streams; often near fresh water [4] [6] [7]. The Baja California Peninsula is one of the longest (1800 km long) and most isolated peninsulas in the world, and it comprehends a heterogeneous array of landscapes and habitats. In this territory, although hummingbird populations exist along environmental gradients, individuals are likely to be limited by particular local conditions [6] [7]. As a consequence, size and genetic diversity of populations could be affected subsequently reducing individuals' fitness. Therefore, understanding the processes of biological diversification and genetic variation for endemic or restricted species like *H. xantusii* is vital to identify evolutionary significant areas for conservation in a scenario of accelerated increase of environmental disturbances (e.g. habitat fragmentation due human activity) [8].

H. xantusii belongs to the family Trochilidae which has the highest specialization rate among nectarivorous birds in the world; it is a medium-sized hummingbird with a remarkably sexual dimorphism, with males having orange bill and colorful plumage [5]. Phylogenetic relationships within the family have recently begun to be studied through molecular data. Most of these studies have focused on the higher classification level (*i.e.* within the family), but given their levels of variability and for a better comprehension of the evolution of hummingbird species, it is necessary to analyze the relationships among and within genera using a similar approach [9]. In this case, we particularly focus on the only endemic hummingbird species of the BCP, the Xantus' hummingbird.

We developed and characterized 16 polymorphic microsatellite loci for *H. xantusii* using 454 pyrosequencing methods. We isolated the loci to be used as a tool in estimating genetic diversity, and for its potential use in further genetic estimations such as gene flow and the measure of population structure across entire distribution of the Xantus' hummingbird. Additionally, we tested the transferability and level of polymorphism of the genetic markers developed in six related species, in order to confirm the utility of the markers for further broader comparative analysis.

2. Material and Methods

2.1. Sample Collection and DNA Preparation

A sample of 25 individuals of *H. xantusii* were collected from three localities; Santiago (23˚48'24"N, 109˚72'30"W), Sierra de la Laguna (26˚04'38"N, 110˚00'68"W) and San Dionisio (23˚55'86"N, 109˚86'55"W), for population characterization. These locations correspond to one genetic population characterized by mitochondrial genes [5]. Additionally, samples from six related hummingbird species (**Figure 1**) were obtained in the field: nine individuals of *Calypte costae* from four localities in the BCP (Santa Gertrudis 28˚05'01"N, 113˚08'65"W; San Ignacio 27˚18'0.63"N, 112˚53'59.32"W; San José de Magdalena 27˚03'47.73"N, 112˚14'37.66"W; and Carambuche 26˚12'53.50"N, 112˚1'35.40"W); five individuals of *Archilochus alexandri* from two localities in the BCP (San Borja 28˚44'36.5"N, 113˚45'13.7"W; and San Isidro 28˚97'58"N, 112˚03'33"W); 10 individuals of *H. leucotis* and six individuals for *Cynanthus latirostris* were obtained from El Fuerte, Sinaloa (26˚25'19.3"N, 108˚37'13.1"W) and Álamos, Sonora (27˚00'53.2"N, 108˚56'51.2"W); five individuals of *Amazilia rutila* and five specimens of *A. candida* from Agua Blanca, Sinaloa (24˚53'28.4"N, 107˚19'28.8"W). Birds were captured with mist nets and blood samples were collected from the tarsus before birds were released. It was verified that no injuries were made to birds during blood sampling.

Genomic DNA was extracted using a salt protocol [10]. A sample of 50 ng of RNA-free, high quality DNA from an individual of *H. xantusii* collected at Santiago, was used for shotgun sequencing by using a 454

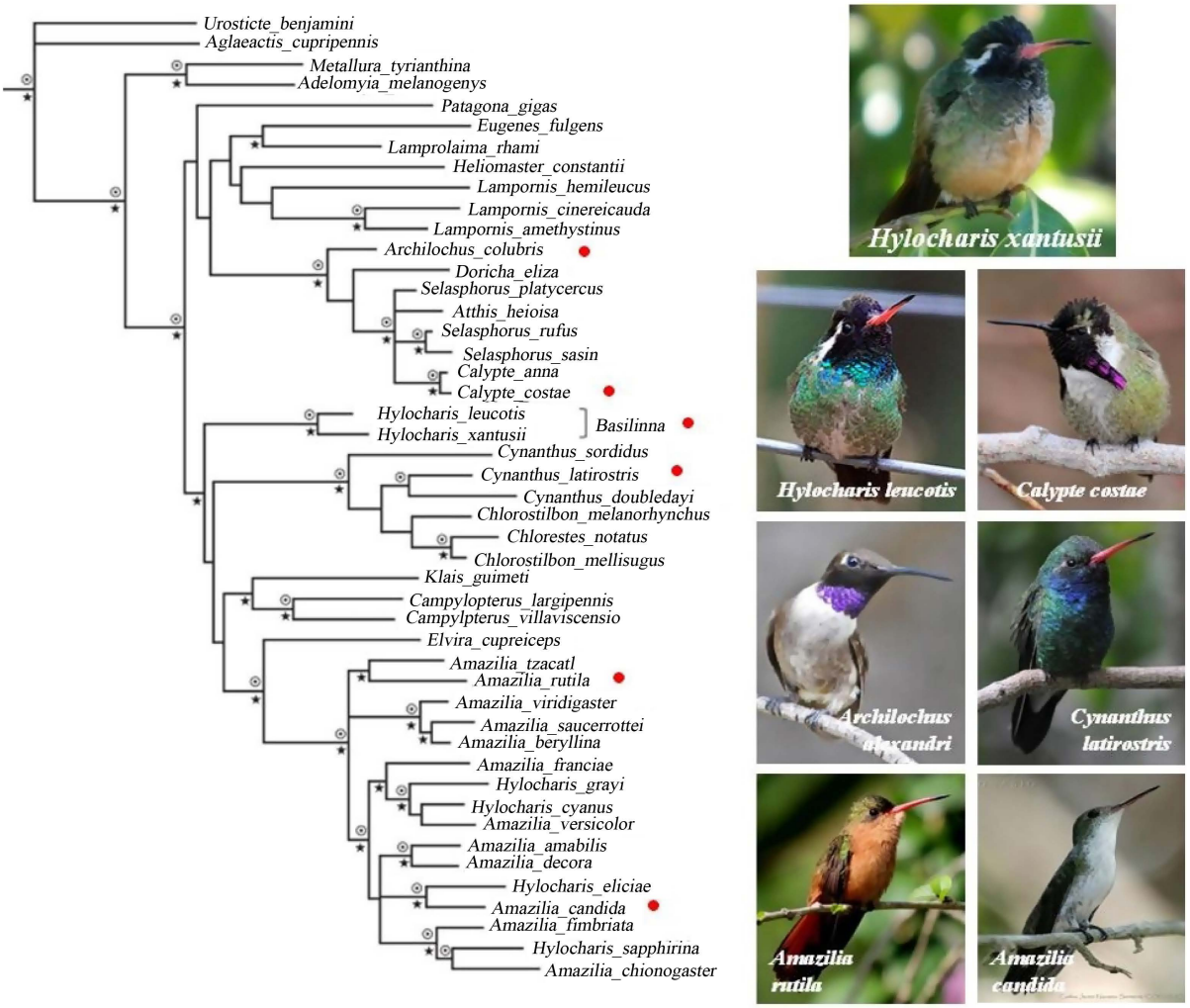

Figure 1. Phylogenetic relationships among hummingbird species adding the microsatellite cross amplification from this work (modified from Hernández-Baños *et al.* [9]). Red dots indicate the phylogenetic position of each species. Picture of *H. xantusii* was taken by CGRS; the rest of the species pictures were taken from the database of Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO; http://naturalista.conabio.gob.mx/, http://bios.conabio.gob.mx/, http://bdi.conabio.gob.mx/).

GS-FLX Titanium instrument (Roche Applied Sciences, Indianapolis, Indiana, USA) at the UCSC Genome Sequencing Center (Santa Cruz, California, USA).

2.2. Microsatellite Loci Identification and Primer Design

All 454 reads were converted to primer design following the methodologies of Abdelkrim *et al.* [3] and Lozano Garza *et al.* [11]. In short, flowgrams were converted to fasta files for the reading of all the sequences; then, Msatcommander [12] was used to identify sequences that included perfect microsatellites with 2 - 6 bp repeat units, and had at least five repeats for tetra-nucleotides. From the set of putative loci, primers were designed using Primer 3 software embedded in QDD [13], using the following specifications: 1) melting temperatures 50˚C - 70˚C with a maximum 2˚C difference between paired primers, 2) PCR product between 90 and 320 bp, 3) GC content > 40%, 4) primers length between 17 and 27 nucleotides, and 5) the primers self-complementarities and complement between them, satisfying the quality criteria used as default parameters. Fifty five primer pairs were chosen for PCR amplification screening and polymorphism test. The loci names were designated with the prefix “Hxan” and a consecutive number (1 - 55). Polymorphic forward primer sequences were labeled with fluorescent dyes (Applied Biosystems, Foster City, California, USA) for automated detection (see below).

2.3. PCR Procedures and Data Analysis

PCR reactions were performed in a 10 µL volume containing about 40 ng of DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.5 - 2.5 µM $MgCl_2$, and 0.5 U Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). The temperature profiles included an initial denaturation at 94˚C for 5 min, followed by 30 cycles at 94˚C for 1 min each and 30 s at the locus-specific annealing temperature (**Table 1**), and a final extension at 72˚C for 5 min. Amplification products were visualized in ABI 310-3730 PRISM® (Life Technologies, Carlsbad, California, USA), using LIZ600 as size standard (Applied Biosystems).

Allele assignation was automated with GeneMapper 4.1 (Applied Biosystems). The minimum peak height acceptance was set to 100 Relative Fluorescence Units (RFU), and the minimum height ratio between peaks for heterozygotes to one third. All peaks assigned were manually checked for allele shape.

Table 1. Summary data for 16 polymorphic microsatellite loci developed for *Hylocharis xantusii*. Includes the GenBank accession number, primer sequences, repeat motifs, annealing temperature (Ta), optimized magnesium chloride concentration ($MgCl_2$), allele size range, number of alleles observed (N_A), observed and expected heterozygosities (H_O and H_E respectively), and statistical significance of deviation from Hardy-Weinberg equilibrium (P_{HW}).

Accession no.	Locus	Primer pair sequence (5'-3')	Repeat motif	Ta (˚C)	$MgCl_2$ [mM]	Size range (bp)	N_A	H_O	H_E	P_{HW}
KU681466	Hxan01	F: TTAAGCACCCCAGTCAAAGG R: CCCAATGTCAGGGATTTTGT	$(AAAT)_6$	65	2.5	220 - 226	4	0.625	0.666	
KU681467	Hxan02	F: AAATCTTCATTTTGCATGCATTT R: CTTTGATGCCATCCCAATCT	$(AAAC)_7$	65	2.5	120 - 156	3	0.542	0.426	
KU681468	Hxan03	F: GGCAGCCCAAATTGCTACTA R: TGTGCTGTTCTCCATCCATC	$(TGGA)_{22}$	65	2.5	117 - 167	7	0.792	0.744	
KU681469	Hxan04	F: CACATTTGTGCTCTGATGGC R: GAGACAACTCAGGCATTCCC	$(ATTT)_6$	70	2.5	169 - 201	7	0.667	0.742	
KU681470	Hxan05	F: CAATGTGCAGTCTCAGGGAA R: CTCCTGTGCTCAAGGGAGAG	$(TGGA)_5$	70	2.5	135 - 178	5	0.292	0.490	***
KU681471	Hxan06	F: GCCAGTGCAGAAGATGGACC R: AGCCTGGCTCCTCCGTAGTC	$(CATC)_5$	60	2.5	132 - 172	5	0.417	0.661	***
KU681472	Hxan07	F: TTCTCACAATGGGAGCCCTG R: GGGAGCAAGTGCATTCAGGA	$(ACAG)_5$	70	2	88 - 156	10	0.792	0.853	
KU681473	Hxan09	F: CTCTGTGATCAGGCTTTTCCA R: AGTGTAGATATAGATGATAGAGACAGA	$(TCTA)_{17}$	66	2.5	141 - 193	10	0.810	0.848	**
KU681474	Hxan11	F: TGGGTTTTCAGTCTGATGGA R: TCTGGGGATTCACATAAGTA	$(CATC)_{15}$	60	1.5	124 - 140	4	0.667	0.510	
KU681475	Hxan12	F: AATGGATGCATGGCTGGTG R: GCCTATGCATTCTCCCAGGC	$(ATGG)_{11}$	60	1.5	110 - 134	6	0.917	0.674	
KU681476	Hxan13	F: CCACTCAGGAAGGCCAAAGA R: GGATGTACCACCAGCTTGCC	$(TGGA)_{13}$	70	2	149 - 185	8	0.500	0.646	***
KU681477	Hxan14	F: CATCACCCTAACAAACCCC R: GCACAGGGTGGGAGTAGTGC	$(ATCC)_{14}$	70	2.5	99 - 176	8	0.522	0.831	***
KU681478	Hxan15	F: GATGTTTCAGGGGAAGTCCG R: TAAATGAGAGCTGCCCCGTG	$(ATCC)_{19}$	66	2.5	78 - 132	9	0.739	0.820	
KU681479	Hxan16	F: GTCACTGCCCAGCAACCACT R: TCAGGGGAGAAGCAGACCAC	$(CAGA)_{10}$	68	2.5	73 - 125	10	1.000	0.810	
KU681480	Hxan17	F: TCAGGGGAGAAGCAGACCAC R: ACTGCCCAGCAACCCACTC	$(TCTG)_{10}$	66	2.5	94 - 154	10	0.875	0.861	
KU681481	Hxan18	F: GCTGGTACCGGAGGTTGATG R: TGGATGAATGACGGAGGAGG	$(CATT)_{13}$	68	2.5	133 - 169	5	0.739	0.681	

**$P < 0.01$, ***$P < 0.001$ = significance of deviation from Hardy-Weinberg equilibrium after 1000 permutation.

Presence and frequency of null alleles were assessed using FreeNA software [14]. Number of alleles per locus (Na), expected and observed heterozygosities (H_E and H_O, respectively) were obtained using GenALEx 6.5 [15]. Deviations from Hardy-Weinberg equilibrium, and linkage disequilibrium between markers were tested using default parameters in GENEPOP 4.2 [16].

3. Results and Discussion

3.1. Identification of Polymorphic Microsatellites Loci

The 454 pyrosequence reaction resulted in 143,625 reads, from which only 815 reads contained a microsatellite motif and were suitable for primer design. Out of the 55 chosen loci for PCR and polymorphism screening, 10 exhibited inconsistent amplification patterns, 14 were monomorphic, and 31 were polymorphic. Within the 31 polymorphic loci, only tretra-nucleotide loci recording successfully more than 50% of the genotypes (**Table 1**), were selected for population characterization.

There was no previous information available for any microsatellite development technique for *Hylocharis xantusii* species. There are just a few hummingbird studies using microsatellite loci [17] [18], but none that had evaluated the effectiveness of microsatellites among closely related hummingbirds by cross-species amplification, or had compared against the results of other birds' species.

3.2. Characterization of Genetic Diversity

GenBank accession numbers, locus ID designation, primer sequences, repeat motifs, number of alleles, observed and expected heterozygosity for the 16 microsatellite loci developed for *H. xantusii* are listed in **Table 1**. The number of alleles per locus ranged from 3 (locus Hxan02) to 10 (locus Hxan07, 09, 16 and 17). The null allele frequency estimates ranged from negligible, less than 0.05 (11 loci) to small, between 0.05 - 0.17 (locus Hxan04-06, 11, 12), according to the ranges proposed in Chapuis and Estoup [14]. Observed heterozygosity among loci ranged from 0.29 (locus Hxan05) to 0.99 (locus Hxan16), with a mean of 0.68; expected heterozygosity ranged from 0.42 (locus Hxan02) to 0.86 (locus Hxan17), with a mean of 0.7. No significant linkage disequilibrium was detected, but five loci (Hxan05, 06, 09, 13, and 14) showed significant deviations from Hardy-Weinberg expectations, after Bonferroni correction.

3.3. Cross-Species Amplification and Polymorphic Levels

Of the six phylogenetically related hummingbird species that were used in this work to validate the effectiveness of the novel microsatellites (**Table 2**), only three showed high levels of polymorphism regarding the number of amplified loci: *Hylocharis leucotis* (87.5%), *Calypte costae* (87.5%), and *Cynanthus latirostris* (75%). The null allele frequency estimates were similar between them, ranging from negligible (less than 0.05) to larger (>0.2) (according to the ranges proposed by Chapuis and Estoup [14]). The number of alleles per locus ranged from 2 to 8; observed and expected heterozygosity were lower than *H. xantusii*, and although no significant linkage disequilibrium was detected, some loci showed statistically significant deviations from Hardy-Weinberg equilibrium, nine for *H. leucotis*, five for *C. costae*, and only one for *C. latirostris*.

C. costae is the only co-distributed species with *H. xantusii*. While *H. leucotis* is the only sister species of Xantus' hummingbird, but with a continental distribution. *C. latirostris* is the most phylogenetic related species to *H. xantusii* and *H. leucotis* [9] (**Figure 1**). These could be the reason for the high polymorphism observed in these species. It has been observed that related species or genetically less distant, have a better microsatellite performance, which means a higher proportion of amplified loci through cross-species amplification [19].

In the other three species (*Archilochus alexandri*, *Amazilia candida*, and *A. rutila*) the number of alleles per locus (two for most of the loci), and the polymorphism values (56%, 44% and 31% respectively), were low compared to other cross-species amplification assays for birds groups [19]. Thus, the possibility of low levels of polymorphism observed being an artifact due to small sample sizes, cannot be discarded (**Table 3**).

3.4. Comparison of Polymorphic Levels between Birds' Species Using Microsatellite Loci

Although it has been observed that avian genomes show a low frequency of microsatellites compared with other organisms [3], many studies have shown that birds have considerable polymorphism to determine measures of

Table 2. Genetic diversity and polymorphism levels using novel microsatellite loci by cross-species amplification. It includes the number and percentage of polymorphism (Polym), number of alleles observed (N_A), and observed and expected heterozygosities (H_O and H_E respectively).

Locus	*H. xantusii* N_A	*H. leucotis* (n = 10) Polym = 14 (87.5%)			*C. costae* (n = 9) Polym = 14 (87.5%)			*C. latirostris* (n = 6) Polym = 12 (75%)			*A. alexandri* (n = 5) Polym = 9 (56.3%)			*A. candida* (n = 5) Polym = 7 (43.7%)			*A. rutila* (n = 5) Polym = 5 (31.3%)		
		N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
Hxan01	4	3	0.200	0.54**	Monomorphic			3	0.167	0.403	2	0.250	0.469	Monomorphic			Monomorphic		
Hxan02	3	2	0.400	0.320	4	1.000	0.735	2	0.167	0.153	Monomorphic			2	1.000	0.500	Monomorphic		
Hxan03	7	2	0.400	0.320	3	0.444	0.593	5	0.667	0.611	2	0.250	0.219	2	0.500	0.375	2	1.000	0.500
Hxan04	7	2	0.300	0.255	2	0.444	0.346	Monomorphic			2	0.750	0.469	Monomorphic			Monomorphic		
Hxan05	5	2	0.300	0.495	2	0.222	0.444	Monomorphic			2	0.250	0.469	Monomorphic			Monomorphic		
Hxan06	5	2	0.000	0.48**	3	0.556	0.66**	2	0.500	0.375	Monomorphic			Monomorphic			Monomorphic		
Hxan07	10	5	0.500	0.715**	4	0.667	0.66*	3	0.667	0.486	2	1.000	0.5*	3	1.000	0.625	2	0.500	0.375
Hxan09	10	5	0.800	0.635	2	0.667	0.444	2	0.333	0.278	Monomorphic			2	1.000	0.500	Monomorphic		
Hxan11	4	3	0.400	0.62**	4	0.444	0.691**	3	0.500	0.625**	-	-	-	-	-	-	-	-	-
Hxan12	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hxan13	8	-	-	-	2	0.444	0.494	-	-	-	-	-	-	-	-	-	-	-	-
Hxan14	8	6	0.400	0.73*	6	0.556	0.778	4	0.833	0.736	3	0.500	0.625*	2	1.000	0.500	2	1.000	0.500
Hxan15	9	6	0.333	0.833*	4	0.444	0.568	5	0.333	0.681	2	0.500	0.375	Monomorphic			2	0.000	0.500
Hxan16	10	3	0.000	0.656**	5	0.286	0.775**	5	0.600	0.720	2	0.250	0.219	4	1.000	0.750	-	-	-
Hxan17	10	8	0.500	0.8*	2	0.500	0.375	8	0.667	0.778	Monomorphic			-	-	-	2	1.000	0.500
Hxan18	5	3	0.300	0.605**	4	0.222	0.561**	2	0.667	0.444	3	0.500	0.531	3	0.500	0.625	Monomorphic		
Mean	6.94	3.71	0.35	0.41	3.36	0.49	0.53	3.67	0.51	0.51	2.22	0.47	0.39	2.57	0.86	0.55	2.00	0.70	0.48

**P < 0.01, ***P < 0.001 = significance of deviation from Hardy-Weinberg equilibrium after 1000 permutation; - = no amplification.

genetic differentiation (see **Table 3**). We compared levels of polymorphism between different birds groups in order to establish if our microsatellite loci of *de novo* isolation and characterization have enough resolution to address questions about the processes driving population divergence and speciation (e.g. gene flow) and about the evolutionary biology of the Xantus' hummingbird (**Table 3**).

For example, in hummingbird species like the wedge-tailed sabrewing (*Campylopterus curvipennis*), which has a complex evolutionary history [17] [18], the analyses of ten microsatellite loci genotyped within 160 individuals (1 to 27 individuals per locality), were enough to evidence the presence of three lineages with no contemporary gene flow.

In general, all genetic studies based on the development of novel microsatellite have shown the effectiveness in the cross-species amplification, regardless of the group, geographic distribution and life history [2] [3] [20]-[27]; even in extreme cases for endemic species like the Japanese wood pigeon (*Columba janthina*), with a high risk of extinction due to its small population size and low genetic diversity [3], seven microsatellites developed for the species and the cross-subspecies amplification, revealed differences in allele frequencies between populations. This showed that despite the low number of alleles observed, the genetic information gathered was sufficient to determine what population faces the major extinction risk.

4. Conclusion

It was found that the isolation of microsatellite loci for *H. xantusii* using next generation sequencing was successful due to the number of polymorphic loci and the number of alleles per locus obtained. These markers are

Table 3. Comparison between the number of microsatellite loci developed and the number of alleles per locus in multiple birds species. Includes the sample size (n) and observed and expected heterozygosities (H_O and H_E respectively).

Species	Distribution	n	loci	Alleles/ locus	H_O	H_E	Reference
Hylocharis xantusii	Endemic of the BCP	25	16	3 - 10	0.29 - 0.9	0.42 - 0.86	This work
Hylocharis leucotis	Southwestern US to Nicaragua	10	14	2 - 8	0.2 - 0.8	0.25 - 0.83	This work
Calyptecostae	Western US and Mexico	9	14	2 - 6	0.22 - 0.9	0.34 - 0.77	This work
Campylopterus curvipennis	Mexico, Belize, Guatemala, Honduras	160	10	4 - 20	0.18 - 0.65	0.30 - 0.64	[17] [18]
Hymenolaimus malacorhynchos	New Zealand's endangered	1	13	2 - 4	-	-	[3]
Garrulax elliotii	Endemic of eastern Himalayas	80	10	2 - 9	0.0 - 0.86	0.07 - 1	[20]
Columba janthina janthina	Endemic islands east Asia	15	7	1 - 7	0.0 - 0.67	0.0 - 0.64	[2]
Columba janthina nitens	Endemic islands east Asia	25	2	1 - 3	0.0 - 0.08	0.0 - 0.08	[2]
Pomatostomus ruficeps	Endemic to Australia and New Guinea	1197	9	11 - 21	-	0.74 - 0.91	[21]
Laruss aundersi	Global and vulnerable	30	9	4 - 15	0.58 - 0.89	0.58 - 0.9	[22]
Varieties of geese	Native of Poland	160	14	3 - 19	0.45 - 0.55	0.38 - 0.51	[23]
Breeds of chickens	Egypt traces	251	29	3 - 20	0.22 - 0.84	0.32 - 0.88	[24]
Columba livia var. *domestica*	Europe, Asia, Africa	22	18	2 - 18	0.23 - 0.91	0.35 - 0.94	[25]
Rhynchotus rufescens	South America	24	6	2 - 12	0.27 - 0.82	0.27 - 0.86	[26]
Ciconia boyciana	North China to southeast of Russia	23	11	2 - 8	0.0 - 0.86	0.22 - 0.85	[27]

- = no data available.

potentially useful for multiple hummingbird species. Moderate to high levels of cross-species amplification were observed across the six hummingbird species (31% - 87.5%), with the best results obtained in closely related species, also showing higher levels of polymorphism (N_A). The availability of these molecular tools enables to address questions integrating population genetics, ecology, conservation, and evolutionary history for *H. xantusii* and for other phylogenetically related species.

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