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Microsatellite markers for Sturnira parvidens (Illumina paired-end sequences), and cross species amplification with other bats of the genus

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Abstract

Background. Sturnira is one of the most species-richness in the Neotropic, and it is found from Mexico, Lesser Antilles to Argentina. Genus forms a well-supported monophyletic clade with at least twenty-one recognized species, and several under taxonomic review. Sturnira parvidens is a widespread frugivorous bat of the deciduous forest in the Neotropics, highly abundant, and a major component in the fruit dispersal to regenerate ecosystems. It can be consider a non-model organism to isolate and characterize polymorphic microsatellites.

Methods. We used a technique based on Illumina paired-end sequencing of a library highly enriched for microsatellite repeats to develop loci for S. parvidens. We analyzing millions of resulting reads with specific software to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites.

Results. We select and test 14 polymorphic (di, tri, and tetra) microsatellites. All markers were genotyped on 26 different individuals from distinct locations of its distributional area. We observed medium–high genetic variation across most loci, but only 12 were functional polymorphic. Levels of expected heterozygosity across all markers was high to medium (mean HE = 0.79, mean HO = 0.72). We probed ascertainment bias in twelve bats of the genus, obtaining null/monomorphic/polymorphic amplifications.

Discussion. Illumina paired-end sequencing system is capable to identify massive microsatellite loci, expending few time, reducing costs, and providing a large amount of data. Described polymorphic loci for S. parvidens particularly, and the genus, could be suitable for further genetic analysis, including taxonomic inconsistencies, parenting/relatedness analysis, and population genetics assessments.

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Keywords: Illumina, Microsatellites, Pal_finder, *Sturnira parvidens*

Introduction

The yellow-shouldered Mesoamerican bat (*Sturnira parvidens*) is primarily associated with lower elevations (0 to 2000 m), found mainly in tropical/subtropical habitats, and ecotones (*Villalobos & Valerio 2002*). *S. parvidens* is found from northern of the Mexican Pacific Slope, and northern Mexican Gulf Slope southward, including Yucatan Peninsula, to Northern Costa Rica (Figure 1) (*Hernández-Canchola & León-Paniagua, submitted*). *S. parvidens* has been caught in the understory or in the subcanopy in tropical and subtropical forests, xeric scrubs, secondary and temperate forest. They like to roost in leaves of forest with advanced successional stages, but their home ranges includes mature and secondary forest (*Evelyn & Stiles, 2003*). It mainly consumes fruits from early stages of plant succession, like pioneer trees (*Cecropia peltata*), pioneer herbs (*Solanum americanum*, *S. torvun*, *S. ochraceo-ferrugineum*, *Capsicum annuum*), or pioneer shrubs (*Piper hispidum*, *P. lapathifolium*; *Olea-Wagner et al., 2007*). This frugivorous species is an important seed dispersal, executing an important ecosystemic role in the restauration of secondary tropical forest. It is considered abundant, which is not found in any risk category but as fragmentation intensifies, the species is particular vulnerable to local extinctions (*Evelyn & Stiles*, *2003*).

Regarding genetic studies, analyzing some mitochondrial and nuclear genes, results showed that Pleistocene climatic oscillations and the complex orogeny of its distributional area shaped the phylogeography of this bat, generating two lowland linages. Both genetic lineages, one in the Western Slope in Mexico, and the other in the Eastern Slope in Mexico and Central America, diverged in haplogroups around c. 0.423 Ma, and demographic expansion was detected later after split-up event (*Hernández-Canchola & León-Paniagua, submitted*). *Sturnira* is the most specious genus of frugivorous bat, due its capability to colonize new areas, complex group showed different genetic linages in distinct stages of diversification (*Velazco & Patterson,*

2013; 2014; Hernández-Canchola & León-Paniagua, submitted).

The genus *Sturnira* involves a highly and complex diversified group of species. This specious group of bats inhabits in all the Neotropic, and it contains three mountain basal species *S. aratathomasi*, *S. bidens*, and *S. nana*. Also, it has been described a clade formed by species that usually inhabits highland mountain forest *S. bogotensis*, *S. burtonlimi*, *S. erythromos*, *S. hondurensis*, *S. koopmanhilli*, *S. ludovici*, *S. magna*, *S. mordax*, *S. oporaphilum*, *S. perla*, and *S. tildae*. Lastly, a group of species that inhabits in lowland tropical forests comprises *S. angeli*, *S. bakeri*, *S. lilium*, *S. luisi*, *S.* new species 3, *S. paulsoni*, and *S. parvidens* (*Velazco & Patterson, 2013*).

Very little is known about molecular markers of *Sturnira parvidens*, our goal was to isolate and characterize polymorphic microsatellite loci for the species by using Next-Generation Sequencing. The making of these markers can be useful to: (1) understand the genetic structure of subpopulations in its distributional range. (2) Identify the human impact in the fragmentation of the populations and assess the divergent linages formed by the genetic drift. (3) evaluate the individual movements in the mosaic-fragmented landscapes, and (4) realize the genetic component in the social structure of the population by assessing relatedness and paternity, etc. We probed cross-species amplification in twelve species of the *Sturnira* genus, under the hypothesis of having a positive ascertainment bias due the phylogenetic relatedness among species (*Crawford et al., 1998*; *Li & Kimmel, 2013*). A suitable cross-species amplification will facilitate similar appointed studies in related bat populations of Middle and South America.

Materials and Methods

We obtained tissue samples for 26 distinct individuals of *S. parvidens* from different localities in its distributional range in Mexico. Matters were proportionate by *Colección de Mamíferos del Museo de Zoología "Alfonso L. Herrera", Facultad de Ciencias-Universidad Nacional Autónoma de México*. Tissue samples were stored individually in 95% ethanol until analysis. We followed guidelines set forth by the American Society of Mammalogists for the use of wild (*Gannon & Sikes, 2007*). Fieldwork was managed under permission of SEMARNAT (Secretaría del Medio Ambiente y Recursos Naturales de Mexico—permit FAUT-0307). Six samples were used to elaborate the enrichment library process, meanwhile the rest were used to standardize protocols and assess polymorphism in microsatellites.

DNA was extracted following instructions of the Qiagen protocol (Blood and Tissue Kit, Cat No. 69504), and in some samples we used the Universal Salt Protocol to extract DNA (*Aljanabi & Martinez, 1997*). An Illumina paired-end shotgun library was prepared by shearing 1l g of tissue DNA using a Covaris S220 and following the standard protocol of the Illumina TruSeq DNA Library Kit. Five million of the resulting reads were analyzed with the program PAL_ FINDER_v0.02.03 (*Castoe et al., 2012*), to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites.

Once positive reads were identified in PAL_FINDER_v0.02.03 they were batched to a local installation of the program MSATCOMMANDER for primer design. We recovered 6790 unique loci (48 hexa, 97 penta, 1260 tetra, 1097 tri and 4288 dinucleotide—Table 1), but only 14 were chosen for PCR trials. We directly labelled forward primers (FAM) for each of the chosen loci. PCR reactions were performed in a 10 µl volume containing 30 ng of DNA, 0.2 mM of dNTP´s, 10 mM of each primer, 1 Taq buffer (Buffer PCR 10x), and 1.0 U of FlexiTaq polymerase. PCR cycling conditions were as follows: initial

denaturation at 95 ºC for 3 min, followed by 30 cycles of 95 ºC for 3 min, gradient temperature for 30 s (range from 56 to 60 ºC), and 72 ºC for 2 min, and extension with 68 ºC for 8 min, and final ending of 4 ºC. Exact annealing temperatures for each primer are given in Table 2. We visualized the PCR products by electrophoresis on 1.5 % agarose gels. Markers were tested for amplification success, polymorphism and specificity in 26 individuals of *S. parvidens*.

The results of the microsatellite profiles were examined using GeneMarker® v. 2.4.2 (SoftGenetics®) and peaks were scored by hand. We obtained the number of homozygotes and heterozygotes by capturing data in an Excel spreadsheet with a .csv extension. We estimated the proportion of polymorphic loci and the average number of alleles per locus by using the GDA software (*Lewis & Zaykin, 2001*). We assessed the observed (H_O) and the expected heterozygosity (H_E), linkage disequilibrium, and Hardy–Weinberg proportions by using Genepop 4.2 (*Rousset, 2008*), and corroborated with Arlequin 3.5 (*Excoffier, Laval & Schneider, 2005*). We used MICROCHECKER to screen null alleles in each locus (*van Oosterhout et al., 2004*). We measured polymorphic information content (PIC) with Cervus 3.0.7 (*Kalinowski, Taper & Marshall, 2007*).

We probed cross-species amplification in tissues of twelve species of the genus: *S. hondurensis, S. burtonlimi, S. oporaphilum, S. mordax*, *S. tildae*, *S. erythromos, S. bogotensis*. *S. magna, S.* new species 3, *S. luisi*, *S. lilium,* and *S. bakeri* (Supplemental Information 1). All polymorphic loci were tested in the mentioned species by using similar PCR conditions. We followed the ascertainment bias hypothesis of broad amplification in similar phylogenetic species (*Schlötterer, 2000*).

Results and Discussion

We obtained a total of 6790 potentially amplified loci (PAL´s), containing perfect, imperfect, and compound

microsatellites (Table 1). Dinucleotide microsatellites were the most abundant (4288), followed by tetra (1260); hexa microsatellites were the less abundant in our lecture (48). PCR reactions showed of the 14 loci tested, two were non-specific or monomorphic, only 12 loci were polymorphic and we were able to get proper amplification (Table 2). Annealing temperature ranged from 56 to 60 °C.

Next Generation Sequencing allowed the project to obtain a large number of microsatellite loci for the target species. This method has been probed for several bat species, and it is becoming a standard method to acquire specific molecular markers (*McCulloch & Stevens, 2011*). This technique is time and cost effective and it is becoming a popular tool for a wide assortment of professionals. Given the natural applicability of microsatellites to solve ecological questions, these molecular markers has emerged as a multipurpose indicator for ecological applications (*Zane, Bargelloni, & Patarnello, 2002*; *Selkoe & Toonen, 2006*). Its applicability strengths academic fields such as population genetics, behavioral ecology, genomics, phylogenies, etc.

We found moderate levels of allelic richness, with an average of 8.8 alleles per locus in the screened wide area of its distribution. Polymorphic information content (PIC) presented values above 0.5 showing a significant content of alleles per locus. Allele frequencies shown a remarkable number of alleles per locus, driving a superior number of valuable loci to be use in different genetic analysis (Supplemental Information 2). No evidence of linkage disequilibrium was found on the analyzed loci. We did not observe loci out of Hardy–Weinberg equilibrium. Levels of HE ranged from medium to high for all markers (mean $H_E = 0.79$, and mean $H_O = 0.72$). There was no evidence of null alleles, but three (Spar05, Spar07, Spar013) showed significant frequencies of null alleles (above 15%--Table 3).

Describers for our microsatellites set the normal standard measures concordant with the evolutionary mutational models proposed for these markers (*Balloux & Lugon-Moulin, 2002*). These indicators provide a straightforward approach to describe genetic variation due the high level of presented alleles. Low allelic richness can affect the accuracy to estimate population genetic parameters, leading to significant errors in genetic diversity of target populations (*Bashalkhanov, Pandey & Rajora, 2009*). Here, we present a novel set of microsatellite loci with the potential to estimate genetic diversity in a non-model species. The proper scenery of describers for our microsatellites may have important implications in the evolutionary biology of the target species, because can be used to develop conservation strategies for Neotropical bats. The use of highly informative microsatellites has been used to assess genetic diversity in a large range of bat populations and to propose measures for its conservation (i.e. *Rossiter et al., 2000*; *Romero-Nava, León-Paniagua & Ortega, 2014*; *Korstian, Hale, & Williams, 2015*).

Amplified microsatellites for *S. parvidens* presented similar levels of polymorphism and heterozygosity found in another bat species (i.e. *Artibeus jamaicensis—Ortega et al., 2002*; *Rhinolophus ferrumequinum—Dawson et al., 2004*; *Desmodus rotundus*- *Piaggio*, *Johnston & Perkins*, 2008; *Corynorhinus* spp.-*Lee*, *Howell & Van Den Bussche, 2011*; *Myotis* spp.-*Jan et al., 2012*; *Carollia castanea—Cleary, Waits & Hohenlohe, 2016*).

Cross-species amplification showed a differential strengthening for the twelve related species (Table 4). *S.* new species3 presented the main number of amplified microsatellites (8), followed by *S. bakeri* (7). *S. mordax* has the less number of amplified loci (4).

The use of microsatellite markers to infer levels of genetic diversity in natural populations is widely distributed.

Molecular markers are not always develop for the target species and the use of microsatellites loci from related species can be accurate. Ascertainment bias limited the microsatellite-based approach due the particular selection of polymorphic markers in the genome, plus the reduced sensitivity of the markers due the phylogenetic constrictions (*Crawford et al., 1998*; *Schlötterer, 2000*; *Li & Kimmel, 2013*). The bias arises in a minor average allele length due the phylogenetic restriction (*Li & Kimmel, 2013*). We tested the potential employ of our markers in related species, founding multilocus heterozygosities inside the *Sturnira* genus. This positive effect suggest using the developed markers to extrapolate genetic diversity in future studies for this highly specious genus; where the past demographic shared histories barely affect the cross-species amplification consolidation.

Conclusions

- We used Illumina Paired-Sequences to developed efficiently microsatellite loci for *Sturnira parvidens*.
- We formed a genomic library to obtain 12 specific and polymorphic microsatellites for this bat.
- We assessed specific PCR conditions to amplify successfully the development of microsatellite loci.
- Microsatellites showed high allelic richness per locus, enquiring their effectiveness for posterior studies (i.e. population genetics, behavioral ecology, etc.).
- Cross-species amplification was effective for the twelve related species, but inexact in several cases.

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226

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Figure 1. - Geographic distribution of *Sturnira parvidens*. It is distributed from the Gulf of Mexico and Pacific slopes in northern Mexico to southeast until Middle America, with a meridional limit in the mountain range of Talamanca in Costa Rica. Map created by E. G. Gutiérrez. Picture of Juan Cruzado.

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Table 1. - Potentially amplified loci (PAL´s) with positive microsatellites found in the enriched library. Perfect, imperfect and compound loci scattered from dinucleotide to hexanucleotide microsatellite forms.

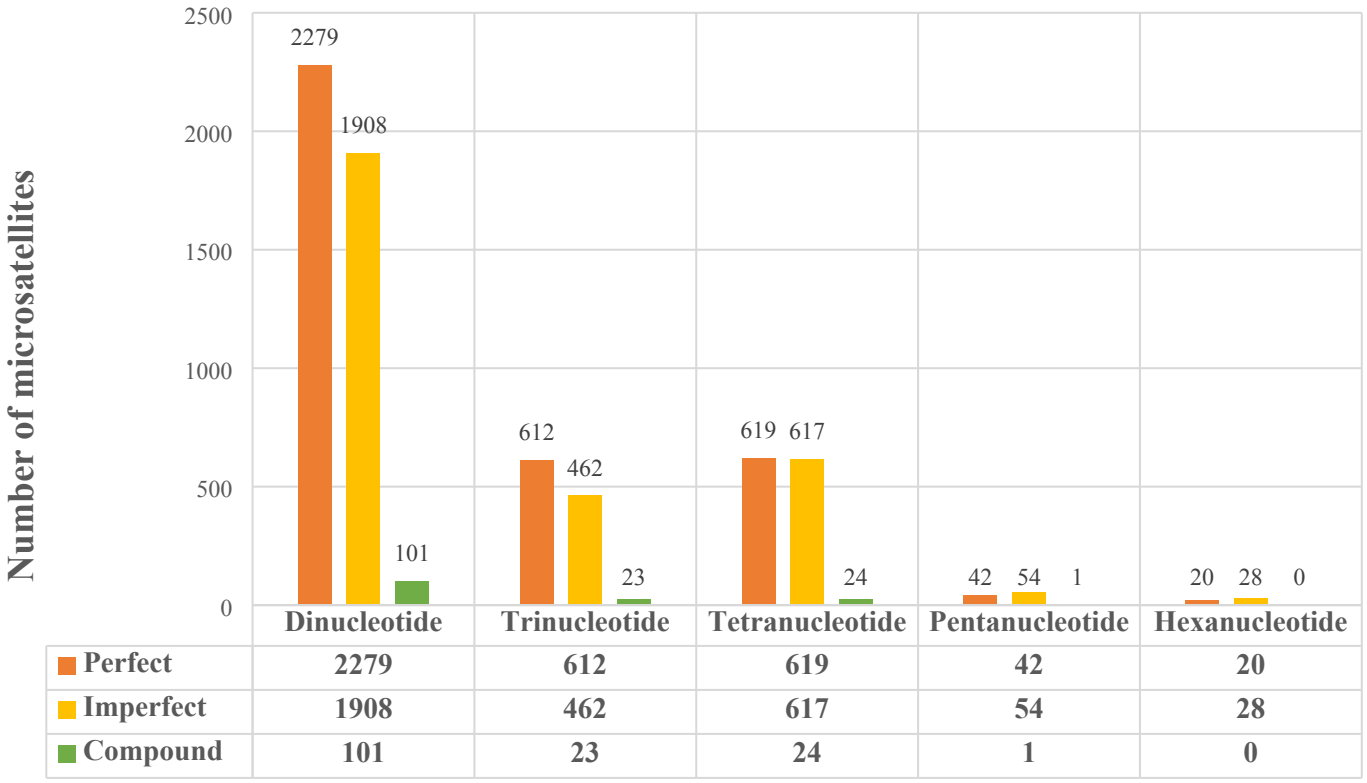

Table 2. - Primer sequences and characteristics of the 14 microsatellites loci isolated for *Sturnira parvidens*.

Locus	Primer (Forward) (5-3')	Primer (Reverse) (5-3')	Motif	Annealing T(°C)

Spar01	6 FAM-TGCCCTGAAGAACTTTGAGC	CCCATACTTCTCCCTCACAGC	AAAG(92)	58
Spar02	6 FAM-AGAAAGAAAGGGAGGGCGG	TTCTTTATGCCCTTTGCTCTAGG	AAAG(104)	60
Spar05	6 FAM-TGCCTGCCTAGTCTGTCACC	AAGCAGTTCCCATCACATGC	ATC(33)	56
Spar06	6-FAM-CCTGGGATGAAGTTTCTGACG	GAATAATGGGAATACCAGAATAAGACG	TTC(30)	✕
Spar07	6 FAM-CTCCCACGGACAATCAACG	CCCAGATTGCTGCCTCTCC	TGC(30)	56
Spar08	6 FAM-GGAGTCTCCTTCATTAAGTGCC	GGATGTGTTGTGAAGATTGTGC	ATT(30)	56
Spar09	6 FAM-AAGTCCATTTCAAGGCTGGG	CCCATCATACCCTCCTTTGC	AC(44)	60
Spar010	6 FAM-TCTGGCCTGAGGTATTTGGG	ACTGTAGCCACTTCCCTGCC	AC(44)	60
Spar011	6 FAM-AAGCCACTGCCTTGTGCC	GACTCTCTGGACATTGGCCC	TC(44)	60
Spar012	6 FAM-GGGAGTGAATGAGAAAGATAAAGTCC	CTGTCATTGCATGGGTTGG	AC(44)	60
Spar013	6 FAM-AAAGATTCCTGGAGATCATACCC	TGAATGTATCCTAGGGCGAGC	AC(42)	60
Spar014	6-FAM-TTTCTCTCACTGTCTAACTCTGCC	AGTCCTGGCAGGTGTGTCC	TC(32)	✕
Spar030	6 FAM-AATGGCACCATATTATTCTACATAGG	CCGTTCTAGGCTCAGTTTCC	ATT (36)	60
Spar040	6 FAM-GACTGAGACAATTGCTTGAGATAGC	GAGTTTCAGGGAGTATTTCAGTGC	ATC(33)	60

Table 3. - Diagnostic characteristics of selected microsatellites. Number of alleles, allelic range, polymorphic information (PI), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), and null alleles.

Locus	GenBank Accession Number	No. alleles	Alleles range (bp)	PIC	Ho	He	HWE	Null alleles
Spar01	KY645946	7	132-236	0.7098	0.941	0.761	>0.05	✕
Spar02	KY645947	6	130-222	0.6455	0.765	0.692	>0.05	✕
Spar05	KY645948	6	124-226	0.6069	0.412	0.699	>0.05	✓
Spar07	KY645949	10	121-226	0.8028	0.824	0.865	>0.05	✓
Spar08	KY645950	11	130-382	0.8052	0.800	0.860	>0.05	✕
Spar09	KY645951	13	134-230	0.8864	0.875	0.933	>0.05	✕
Spar010	KY645952	12	132-236	0.8698	0.882	0.919	>0.05	✕
Spar011	KY645953	8	124-222	0.8125	0.588	0.863	>0.05	✕
Spar012	KY645954	8	128-214	0.7068	0.750	0.772	>0.05	✕
Spar013	KY645955	10	124-220	0.8577	0.500	0.867	>0.05	✓
Spar030	KY645957	6	133-169	0.7088	0.741	0.735	>0.05	✕
Spar040	KY645958	6	124-190	0.6721	0.662	0.669	>0.05	✕

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Locus	*S. hondurensis*	*S. burtonlimi*	*S. oporaphilum*	*S. mordax*	*S. tildae*	*S. erythromos*	*S. bogotensis*	*S. magna*	*S.* newspecies_3
Spar01	✕	✓p	✕	✕	✕	✕	✕	✕	✓p
Spar02	✓p	✕	✓*	✕	✕	✕	✕	✓*	✓*
Spar05	✓p	✓p	✓*	✓*	✓*	✕	✕	✓*	✓*
Spar07	✕	✕	✕	✕	✕	✕	✕	✕	✓p
Spar08	✓*	✓p	✓*	✓p	✓*	✓*	✓*	✕	✓p
Spar09	✕	✓p	✓*	✓*	✓*	✓*	✓*	✕	✓p
Spar10	✕	✓*	✓*	✕	✓*	✓*	✓*	✓*	✓p
Spar11	✓*	✓p	✓*	✓p	✓*	✓*	✓*	✓*	✓*
Spar12	✓m	✕	✓*	✕	✓*	✓*	✓*	✓*	✕
Spar13	✕	✕	✓*	✕	✓*	✓*	✓*	✓*	✕

257 **Table 4. -** Cross-species amplifications of the designed primers for *S. parvidens*. We followed same PCR conditions in the

twelve related species. (✗) no positive amplification, (✓p) positive polymorphic amplification, (✓m) positive monomorphic amplification, (✓*) polymorphism not probed because inexact PCR conditions.