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SHORT COMMUNICATION

Development of ten microsatellite markers from the keystone mistletoe *Tristerix corymbosus* (Loranthaceae) using 454 next generation sequencing and their applicability to population genetic structure studies

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Abstract *Tristerix corymbosus* (Loranthaceae) is a keystone mistletoe from the South American temperate rainforests. As most mistletoes, *T. corymbosus* relies on biotic pollination and seed dispersal, which may cause population structure. For a better understanding of its ecology, we isolated and characterized ten polymorphic microsatellite loci for this species. We used 454 Next Generation Sequencing to build a microsatellite library from a high quality DNA sample. We tested 90 sequences from which we obtained ten polymorphic markers. In order to assess their variability, the novel markers were tested in 48 individuals from three locations of the Valdivian Coastal Reserve in Chile. We also estimated genetic differences between pairs of populations using the F_{ST} statistic. The mean number of alleles per locus in the 48 individuals studied was 7.1 (2–17 alleles per locus). The observed and expected heterozygosity ranged from 0.298 to 0.634 and from 0.310 to 0.881, respectively. There were genetic differences among the three populations assessed, according to the F_{ST} values (ranging from 0.048 to 0.100, all significant) and, the number of alleles per locus ranged from 3.9 to 5.1. These are the first microsatellite markers developed for *T. corymbosus*, and they arise as a powerful tool for studying population structure, genetic diversity and gene flow at the landscape scale, along its distribution.

Keywords Genetic diversity · Genetic marker · Microsatellites · *Tristerix corymbosus*

Introduction

Many mistletoes often play a key role in forest ecosystems by providing food resources to many vertebrates during scarcity periods [13]. Due to their parasitic life form, mistletoe populations depend on host population spatial arrangement and biotic vectors for pollination and seed dispersal to guarantee their reproductive success [8]. Such dependence on host and biotic vectors is a potential cause of population structuring, by limiting gene flow at the landscape level [7]. *Tristerix corymbosus* (Loranthaceae) is a diploid hemiparasitic mistletoe widely distributed along southern South America (30°–42°S), in Argentina and Chile [3]. This mistletoe depends almost exclusively on the hummingbird *Sephanoides sephaniodes* for pollination [2], and on the frugivorous bird *Mimus thenca* (between 30° and 37°S) and the relict marsupial *Dromiciops gliroides* (37°–42°S) for seed dispersal [4], constituting a very specialized mutualistic system. *Tristerix corymbosus* reaches its flowering peak during the winter months (flowering season extends from March to October), and its fructification peak between January and February (fruiting season extends between November and March). Because of that, this mistletoe plays a key role in the South American

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temperate rainforests by providing food for *S. sephaniodes* during winter time, as well as providing sugar-rich fruits for birds and mammals in summer [1]. Furthermore, this plant is from ethnobotanical importance as local people use it to treat different illnesses, depending on the host plant that the mistletoe parasites as it captures different secondary metabolites on each case [9].

Despite its specialization and ecological importance, many aspects of *T. corymbosus* ecology remain unknown, particularly those related to population structure, genetic diversity, and gene flow. The only population study in *T. corymbosus* involving genetic data is using chloroplast DNA sequences [3], which is limited for population studies because they are maternally inherited and are more suitable for phylogeny studies due to their low variability [11]. Thus, the development of microsatellite markers for *T. corymbosus* may largely contribute to our understanding of its ecology, by providing information on its population structure, genetic diversity, and population effective size. We can also improve our understanding of the rate and direction of migrations at the landscape scale, which may improve habitat restoration plans by taking advantage of the indirect positive effects that this mistletoe may have on its host plants [6]. Here we present the isolation and characterization of 10 microsatellite loci for *T. corymbosus*, which were tested in 48 individuals from three different sample groups.

Materials and methods

Sampling site and protocol

Genetic samples of 48 *T. corymbosus* individuals were collected in a private protected area of southern Chile, the Valdivian Coastal Reserve (39°57′S, 73°34′W), which presents a complex habitat mosaic composed by old- and second-growth native forest stands, and a transformed habitat composed by abandoned *Eucalyptus globulus* plantations with abundant native understory vegetation. Within the *Eucalyptus*-dominated habitat, we sampled *T. corymbosus* plants in three specific locations: T1 (39°58′01.62″S, 73°38′54.55″W), T2 (39°58′25.31″S, 73°39′08.89″W), and T3 (39°58′54.01″S, 73°39′12.71″W) (Fig. 1). Each sampled mistletoe was tagged and georeferenced. Samples were collected from March 2013 to February 2014. Young leaves were immediately stored, dried, and preserved in hermetic Ziploc bags with silica gel until DNA extraction.

DNA isolation and sequencing

Genomic DNA was isolated from the 48 samples collected in the field using a DNEasy plant mini kit (QIAGEN, Valencia, California, USA). We used ~10 to 20 mg of dry plant material, previously ground using a Mini-Beadbeater-96 device (BioSpec Products, Bartlesville, Oklahoma, USA). DNA sample quantity was measured with a Nanodrop Spectrophotometer (Thermo Fisher, Wilmington, Delaware, USA). The quality of a sample with a high-quantity of DNA was checked with the Bioanalyzer Agilent Model 2100. The enriched library was built using a range of 500 ng to 1 μg of DNA, the GS Rapid Library Preparation kit and a 1/4 plate of a 454 Roche GS Junior was used to sequence a part of the genome. The NGS was performed at OMICS Solutions (http://omics-solutions.cl). Sequencing generated a total of 14.3 Mb of quality-filtered data, corresponding to 29,474 non-redundant reads. The MISA 4.0 software (http://pgrc.ipk-gatersleben.de/misa/) was used to search for repeated motifs (di and tetra), and primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). A total of 5582 reads with microsatellites were detected, resulting in 2352 reads with primers. The complete microsatellite library generated is available online (Appendix 1).

Polymorphism assessment

Genomic DNA from four single individuals was used to test 90 microsatellites. The polymerase chain reaction (PCR) amplification mixtures (10 μl) contained 80 ng template DNA, 0.10 μM of forward primer and 0.25 μM of reverse primer, 250 μM of each dNTP (Applied Biosystems, Foster City, CA), 5 mM $MgCl_2$, 1 μl 10 × PCR buffer and 1 U Taq Polymerase (Invitrogen, Carlsbad, CA). Cycling conditions consisted of an initial denaturing step of 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at the specific annealing temperature, 1 min at 72 °C, and a final elongation step at 72 °C for 10 min. For the markers that showed a reliable amplification in the agarose gel electrophoresis, polymorphism was evaluated in an Applied Biosystems 3100 automated sequencer (Foster City, California, USA) with the forward primer of each locus containing 0.16 μM of the fluorescent dye (FAM, VIC, NED and PET) with the M13 universal sequence primer (CACGACGTTGTAAAACGAC). PCR products were genotyped in the sequencing core at Pontificia Universidad Católica de Chile (PUC), using the internal size standard LIZ 500 (Applied Biosystems, Foster City, California, USA).

Descriptive statistics and population structure

GENEMARKER software (http://www.softgenetics.com/GeneMarker.html) was used to build the matrix with allelic data. The MICROCHECKER software [12] was used to identify possible genotyping mistakes, large allele dropouts

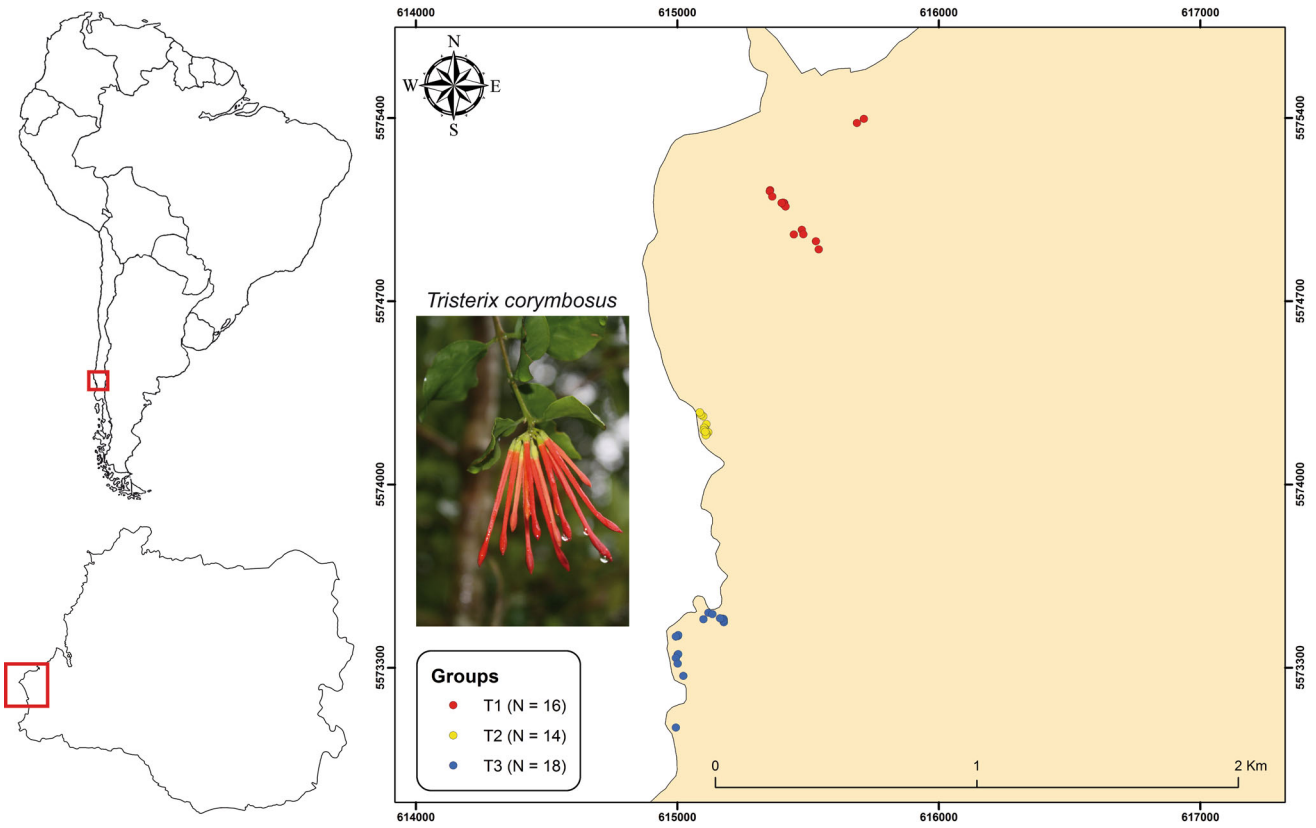

Fig. 1 Map of the study area. *Upper left* Los Ríos Region location in South America, *bottom left* study area location in Los Ríos Region, *right* sampling points in the study area and a picture of the study species

and the presence of null alleles in the microsatellite data. Observed (Ho) and expected (He) heterozygosity and number of alleles (Na) were estimated in the GENETIX software [5] for the 48 individuals altogether. Linkage disequilibrium was estimated for all pairs of loci in each group with 5000 permutations. Then, we estimated Ho, He, deviation from the Hardy–Weinberg equilibrium (HWE) and Na values of each locus in each mistletoe group. We used the F_{ST} based on the multilocus allelic frequency gathered from the individuals collected at each population to estimate genetic differences between pairs of populations [14]. Individual permutations among populations (5000 permutations) were used to estimate the statistical significance of this index. To reduce Type I error, $\alpha = 0.01$ was used in these paired F_{ST} comparisons. F_{ST} calculations were also estimated using GENETIX.

Results and discussion

Polymorphism assessment

From the 90 microsatellite loci tested, a total of 40 showed amplification, the PCR products of these microsatellite markers were genotyped in 11 individuals of *T. corymbosus* and a total of 11 markers were polymorphic: TRIS_29, TRIS_30, TRIS_36, TRIS_46, TRIS_59, TRIS_68, TRIS_69, TRIS_76, TRIS_80, TRIS_82 and TRIS_84. These 11 loci were genotyped in the rest of the samples.

Descriptive statistics and population structure

No genotyping mistakes or large allele dropouts were detected, but the locus TRIS_82 showed the presence of null alleles in the three locations sampled, being excluded from the rest of the analyzes. No significant genotypic linkage disequilibrium was detected indicating that the loci are probably not closely linked on chromosomes and, consequently, the loci may be considered as independent markers. The characteristics of the 10 microsatellite markers are described in Table 1. The Ho and He ranged from 0.298 to 0.634 and from 0.31 to 0.881 respectively, while the allele number per locus ranged from 2 (TRIS_36 and TRIS_68) to 17 (TRIS 30) with an average of 7.1 alleles per locus. Population T1 showed Ho and He values ranging from 0.063 to 0.693 and from 0.061 to 0.809 respectively, departures from the HWE for the loci TRIS_59, TRIS_76, TRIS_ 80 and TRIS_ 84 were

Table 1 Summary of the characteristics for the ten microsatellites developed for *Tristerix corymbosus*

Locus	Primer sequence (5′-3′)	Repeat motif	Ta (°C)	n	Ho/He	F_{IS}	Na	Size range (bp)	GenBank accession number
TRIS_29	F: TTTCTGCAAGTATACTACTAC R: CTAACATTGATAGAAAGAAC	(TA)8	54	47	0.468/0.496	0.067	4	159–167	KU207086
TRIS_30	F: AACTAATGTAAAACTGTCAC R: GTGATATAAGCAGTTCATAT	(TC)8	54	41	0.634/0.881	0.292*	17	220–268	KU207087
TRIS_36	F: GTATGCTCTATACAAGAAAC R: CATATATCACTTTTGAAGG	(AATA)8	54	46	0.500/0.471	−0.050	2	181–185	KU207088
TRIS_46	F: GTTACTAGTTCACTAATACCA R: TAGTAGTAGCATCACACATAG	(TA)11	54	46	0.522/0.666	0.227*	13	346–390	KU207089
TRIS_59	F: CTAATTAGTATTAGGGATGA R: GTGTATGTGTGTATGTATTTC	(AC)8	54	48	0.354/0.499	0.300*	4	445–455	KU207090
TRIS_68	F: GTAGTATTGAATTTACAACC R: GTTAGAGATTTATGGAAGA	(CT)6	54	47	0.298/0.310	0.049	2	261–263	KU207091
TRIS_69	F: CTCTATATTTAGACAGGGTA R: TACTATAATCTCATATGGGA	(GA)8	54	48	0.396/0.456	0.141	3	279–285	KU207092
TRIS_76	F: GCTGTATATATGTGATTTGT R: AACATATAATACTCATGGAG	(TA)8	54	41	0.488/0.651	0.262*	3	354–358	KU207093
TRIS_80	F: GAAGTCTTAGAACCTGATAT R: TAGCTAAAACTAGAGACATAC	(AT)9	54	43	0.442/0.824	0.473*	10	372–398	KU207094
TRIS_84	F: TCTCGTAGTCTAATTACACTA R: CTCACTTATTGATATCATTC	(AT)6	54	43	0.326/0.547	0.415*	13	406–488	KU207095

Ta annealing temperature, *n* number of individuals analyzed, *Na* number of alleles detected, *Ho* observed heterozygosity, *He* expected heterozygosity, F_{IS} individual F-statistic accounting for deviations in the observed number of heterozygotes

* Indicates departure from HWE ($P < 0.05$)

observed; while population T2 showed Ho and He among 0.286–0.833 and 0.293–0.760 respectively, and only locus TRIS_84 showed departures from the HWE. However, population T3 showed deviations from the HWE in three loci (TRIS_30, TRIS_ 76, TRIS_80 and TRIS_84) with Ho and He values ranging from 0.250 to 0.611 and from 0.219 to 0.799 respectively. Only locus TRIS_84 showed consistently departures of HWE for the three populations. Population T3 showed the highest number of alleles (mean number of alleles per locus = 5.1) followed by population T2 (mean number of alleles per locus = 4.0), while population T1 showed the lowest number of alleles (mean number of alleles per locus = 3.9) (Table 2). The global F_{ST} of the locations sampled in the Valdivian Coastal Reserve was 0.0782 ($P < 0.0001$), indicating significant population structure. Pairwise analysis suggested that the three sampled groups represent independent and different populations: F_{ST} T1–T2 = 0.100, $P < 0.01$; F_{ST} T1–T3 = 0.088, $P < 0.01$; F_{ST} T2–T3 = 0.048, $P < 0.01$.

In summary, ten polymorphic microsatellite DNA loci have been specifically developed for *T. corymbosus* in this study. Due to its characteristics, the microsatellites (high mutation rate, co-dominant inheritance and selectively neutral) are excellent markers to analyze the genetic structure at the individual and population level [10]. The microsatellites described here showed moderately high levels of polymorphism arising as a useful tool to perform studies about the population structure, gene diversity and migration rate in *T. corymbosus* in order to improve the biological information for future conservation efforts for this species and their hosts. This is particularly relevant for degraded habitats, where this mistletoe thrives forming dense aggregates, which may be potentially restricted gene flow at the landscape scale.

Table 2 Population genetic parameters for each locus of *Tristerix corymbosus* in three populations examined

Locus	n	T1			n	T2			n	T3		
		Ho/He	F_{IS}	Na		Ho/He	F_{IS}	Na		Ho/He	F_{IS}	Na
TRIS_29	16	0.188/0.342	0.477	2	13	0.769/0.536	−0.403	3	18	0.500/0.545	0.111	4
TRIS_30	13	0.693/0.840	0.215	8	12	0.833/0.76	−0.053	7	16	0.438/0.772	0.459*	12
TRIS_36	16	0.625/0.492	−0.240	2	14	0.643/0.497	−0.258	2	16	0.25/0.219	−0.111	2
TRIS_46	15	0.400/0.549	0.303	5	13	0.539/0.618	0.168	7	18	0.611/0.704	0.159	7
TRIS_59	16	0.313/0.607	0.510*	3	14	0.429/0.538	0.239	4	18	0.333/0.290	−0.121	3
TRIS_68	15	0.200/0.278	0.311	2	14	0.357/0.293	−0.182	2	18	0.333/0.346	0.064	2
TRIS_69	16	0.063/0.061	0.000	2	14	0.643/0.477	−0.315	2	18	0.500/0.495	0.019	3
TRIS_76	13	0.308/0.589	0.508*	3	12	0.833/0.663	−0.215	3	16	0.375/0.646	0.500*	3
TRIS_80	15	0.467/0.809	0.451*	7	12	0.500/0.691	0.316	5	16	0.375/0.799	0.553*	9
TRIS_84	15	0.400/0.651	0.415*	5	14	0.286/0.421	0.900*	5	14	0.286/0.469	0.100*	6
Overall		0.365/0.522	0.332*	3.9		0.583/0.550	−0.021	4.0		0.400/0.529	0.272*	5.1

Overall represents the multilocus estimations and average number of alleles per locus for each population

n number of individuals analyzed, *Na* number of alleles detected, *Ho* observed heterozygosity, *He* expected heterozygosity, F_{IS} individual F-statistic accounting for deviations in the observed number of heterozygotes

* Indicates departure from HWE ($P < 0.05$)

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