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Microsatellite markers in Spanish lime (Melicoccus bijugatus Jacq., Sapindaceae), a neglected Neotropical fruit crop

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SHORT COMMUNICATION

Microsatellite markers in Spanish lime (*Melicoccus bijugatus* Jacq., Sapindaceae), a neglected Neotropical fruit crop

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Abstract Spanish lime (*Melicoccus bijugatus* Jacq.) is a Neotropical fruit tree cultivated, mainly, in orchards for self-consumption or local sale. The genus *Melicoccus* includes other nine species with edible fruits, some of these species are at risk of extinction. Like for the vast majority of tropical fruit trees, there is no information on the genetic diversity of Spanish lime and its related species, and this is mostly due to the lack of molecular markers. The objectives of this study were to present the first microsatellite markers developed for Spanish lime, testing its usefulness on a sample of cultivated accessions, as well as its transferability to Huaya India (*M. oliviformis*). To do this, we performed high-throughput sequencing of microsatellite-enriched libraries of Spanish lime using Roche 454, assembled 9567 DNA contig sequences and identified 10,117 microsatellites. After screening 384 of those microsatellites on four DNA samples, 31 polymorphic markers were used to screen 25 accessions of Spanish lime and five of Huaya India collected in Yucatan, Mexico. Genetic diversity was low in Spanish lime (A = 20.61, H_E = 0.38) and similar for both sexes of this species. Neighbor-Joining and PCoA analyses clearly discriminated between the two *Melicoccus*

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species studied. Nine of the markers showed unique alleles for Huaya India. The set of microsatellite markers developed has a great potential to generate information in relation to conservation genetics, improvement of elite cultivars and breeding programs for Spanish lime and related species.

Keywords Huaya India · *Melicoccus oliviformis* · Mexico · Yucatan state · SSR markers

Introduction

Tropical regions harbor a great diversity of fruit trees that for centuries have provided food and medicine to humankind (Normah et al. 2013; Paull and Duarte 2012). Usually, more than 90% of the fruit tree production in tropical areas is consumed locally as fresh fruit, though there has been a steady increase in demand of tropical fruits in world markets, as people become aware of their nutritional value and the health benefits associated to the consumption of these fruits (FAO 2010).

The genus *Melicoccus* (Sapindaceae) includes 10 species of Neotropical fruit trees. Its distribution range extends from the Yucatan peninsula in Mexico to South America, plus an isolated species in the Dominican Republic (Acevedo-Rodríguez 2003). Spanish lime (*Melicoccus bijugatus* Jacq.) and Huaya India (*M. oliviformis* Kunth.) are the most widely distributed species since their fruits, produced on female dioecious trees, are consumed in tropical areas around the world (Acevedo-Rodríguez 2003). Spanish lime is consumed as fresh fruit, mainly, but also as preserves, or in drinks and alcoholic beverages (Martin et al. 1987). Though some elite cultivars of Spanish lime have been generated, it is mainly cultivated in orchards of villages of South America, Central America, the Caribean and the Yucatan peninsula in Mexico (Morton 1987), and its cultivation has not reached international marketing levels such as lychee (*Litchi chinensis* Sonn.) and rambutan (*Nephelium lappaceum* L.), species of the Sapindaceae family but of Asian origin. Ethnobotanical information about Spanish lime includes the use of both seeds and pulp for medicinal purposes and multiple biologically active compounds have been found in the seed embryo, including flavonoids such as procianidine, epicatequine, catequine, epigalocatequine, naringenin and their derivatives phloretin, floridzine, quercetine, miricetin and resveratrol, all these compounds are used to treat diseases, e.g., parasite infections, diarrhea, fever and sore throat (Bystrom 2012). So, the medicinal value of Spanish lime could be as important as other Sapindaceae species (lychee, longan, rambutan) well known in Asia.

Currently, there are no studies on the genetic diversity of Spanish lime using co-dominant molecular markers, and the same is true for many tropical fruit trees. Though single-nucleotide polymorphisms markers (SNPs) are becoming increasingly popular given their abundance in genomes and the availability of high-throughput genotyping technologies, the microsatellites continue to be the most commonly used molecular markers. Because of their reproducibility, multi-allelic and co-dominant nature, microsatellites have multiple applications such as population genetics, gene mapping, performing marker assisted selection, and constructing linkage maps (Vieira et al. 2016). However, the development of microsatellite markers continues to be the limiting factor to study minor crops, such as tropical fruit trees. Our objectives were to develop the first microsatellite markers for Spanish lime, testing its usefulness on 25 cultivated accessions of Yucatan, Mexico, as well as its transferability to Huaya India.

Materials and methods

Two samples of Spanish lime from the collection at USDA-ARS Tropical Agriculture Research Station (TARS): accessions ‘Sasa’ (TARS 18206) and ‘Doña Santos’ (TARS 18219) were used to develop microsatellite markers for this species. DNA was extracted from leaves using DNeasy Plant Maxi kit (Qiagen, Valencia, CA). Microsatellite-enriched libraries were prepared according to Techen et al. (2010) with adaptations of the method for high-throughput sequencing as previously described in Arias et al. (2015). The libraries were sequenced in a Roche 454 GS-FLX (Roche, Branford, CT) using GS Titanium Sequencing kit XLR70 pico-titer plate (Roche, Branford, CT) for 200 cycles. Sequences were assembled with 454 gsAssembler version 2.0 (Roche, Branford, CT), microsatellite searched using SSRFinder (Sharapova et al. 2002), and primers were

designed by Primer 3 (Rozen and Skaletsky 2000) using stringent conditions as described for other species (Arias et al. 2015).

Of the total markers obtained, a subset of 31 polymorphic markers (Table 1) were used for fingerprinting 30 *Melicoccus* accessions and test its usefulness in the species, as well as its transferability to Huaya India. To do this, 25 cultivated accessions of Spanish lime (15 female plants, 10 male plants) and five cultivated accessions of Huaya India were collected at flowering in Yucatan, Mexico (Table 2). Fingerprinting was performed as described by Arias et al. (2015) and briefly summarized here. An adaptor sequence (5′-CAGTTTTCCCAGTCACGAC-3′) was added to the 5′ end of the forward primers. This forward primer sequence was then labelled with 6-carboxy-X-rhodamine (ROX) dye (IDT-Technologies, Coralville, IA) and used for amplification of

Table 1 Marker name, forward and reverse sequences, size range and allele number for 31 microsatellite markers screened on 30 accessions of *Melicoccus* spp. from Yucatan, Mexico

Marker	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Size range	No.
MB0695a	TTAACTCAACTTTCCGACAGCAGC	TCTTGGTAGAGAAGTGAAGCCAGC	165–211	4
MB0743a	AAAGGAGACCAAACCCTAAATTGC	GTAATAGGGAATGATTCGGTGGTG	105–292	9
MB1842b	TCTCGTCTTCTTCTTCAATAGAACCAAC	CACTGGGATTTCTGGTGTACTTCC	144–157	3
MB1935a	TGATGATGAGGCGATAGAATATGG	AACATCCCAAACTTCTTCAACACC	130–524	3
MB2129a	ACGATGTTTTTGCTGTTGACTTTG	TTTCATAAATGTTACGCATGTCACG	105–165	12
MB2361a	TTGCAAGTGCTAGAAAATCCATTG	TCCGTTGCTACATCTTCTTCTTCAC	125–154	4
MB2400a	CATTCAATGCAATAAGGAGAAGCTG	GTAATCAATGTGGGATGTGATTGG	231–235	2
MB2695a	GGCAAGCCAACTGTAGGATTTATG	ACAGGTTCCTCAACTGCTGTGAC	169–172	2
MB3227a	*AGTCTGCAAAAGTCGCAAAAATG*	*GTTTGTAGCATGACTGGACCAATG*	*147–279*	*4*
MB3400a	CCCTCATCATCGTCGTAGGTTATC	TTTCAGAAAGCGAAATGAATACAAAAG	135–212	4
MB3615a	GTGGTAGAAGGATCAGGGAGAGAG	CTCGTCATCTCTTTTCTCTCATGG	139–231	6
MB3630a	GGTTTTTCCAGGCTCCTAGATCG	TTCATAGCTATTTGTGACACCCCG	131–484	10
MB4008a	AATCCCACTCTTTGATCCAGTTTG	TTAAAACAACACAACCACAGCCAC	128	1
MB4119a	AGGGAAGAAGGAATTGTGGAAAAG	TGTCGTCGTCTGCTTTCTCTATTG	102–175	5
MB4221a	*GATACTTGGTGGAATAGAAGGGTGG*	*CCACTGCAGCAATCTTAAACACAC*	*112–124*	*3*
MB4366a	TAGGCTGAATTTGGTGGAGAGAAG	TGGTGGGATGTACCAATCTTAATTC	171–176	2
MB4380a	*TCAAAAATTTCAAAACCCCTTCTTC*	*TCATCAGTAGGGTGGAACCTGAAC*	*180–203*	*2*
MB4821a	*ATGTCATCTTCCTCTTCCCTTTCC*	*CTATTGGAGTTGGAGTCACCGAAG*	*106–184*	*6*
MB4847a	GAGGACAGAGAAGAGCAAGAGTGG	CACATTCGAACCTACTAACCAGCC	166–193	3
MB4915a	GTTAAGGTTTTCAATGTGGGAAAGG	GGCTCTCAAAATGACAGAAAACTCC	129–178	3
MB4947a	TGACTAAACCTTTCAGGCTGATCC	ATCGTCACCAACGAAAGTTGC	156–179	3
MB5054a	*AGAGCGCCTAGAAGCAGAAGAAG*	*CATACACTTCCACATTCGTCCTTG*	*140–149*	*3*
MB5123a	*CCAGTTTACATAATGCAAGTTCCG*	*GCACATGAAAGCTAACCAAAACAC*	*129–143*	*5*
MB5617a	*TTTGTGGATGGTTGACTTTTGAAG*	*AACAACAAACATGCAAACAAAACC*	*135–149*	*4*
MB5801a	TGTGATGGAGGGTTTTGTTTTCTC	AACGATATTCGTTTCTTGGCGTC	168–194	6
MB5932a	AACTGAAACACAACCTTTCACTTCATC	AGCATGTGGTCCGATAATCTTTTG	101–116	4
MB6181a	*CCCTCTTCCTCTCCCTATCAGTTC*	*ACAGAACTTGAAAGCCTGATTTGAC*	*96–106*	*2*
MB6289a	CTTGGTTCAATCTTGAAGTCCCAC	GAGTAAAAAGTAGAGATCAACTCCCACG	100–116	4
MB6464a	GGAGTTAGACAACCAAATAAATTAACCG	ATCCTCCACATCAAAAACCATTTC	161–193	5
MB6579a	ACAAAACAGAGCTGACTCCAAACC	TTGGTGTTTCTGGTCATGAAAATG	157–182	6
MB7267a	*GTCAATACGTCCAAAATCCATGC*	*GCATTTGCTATGTTGCTTGAAGAG*	*175–191*	*4*

Size range: amplicons in base pairs; No.: number of alleles observed at each locus. Italic format indicate the nine markers that discriminated *Melicoccus oliviformis*

Table 2 Data of the accessions of *Melicoccus bijugatus* and *M. oliviformis* collected in Yucatan, México

Accession	Species	Village	Coordenates		Female	Male
ACAN_F	*M. bijugatus*	Acanceh	20°48′46″N	89°27′13″O	X	
BUCT_M	*M. bijugatus*	Buctzoz	21°12′06″N	88°47′34″O		X
BUCT_F	*M. bijugatus*	Buctzoz	21°12′06″N	88°47′34″O	X	
BUCT_M	*M. bijugatus*	Buctzoz	21°12′06″N	88°47′34″O		X
CALO_F	*M. bijugatus*	Calotmul	21°01′08″N	21°01′08″N	X	
CHOC_F	*M. bijugatus*	Chochola	14°35′00″N	91°27′00″O	X	
CHUM_M	*M. bijugatus*	Chumayel	20°25′42″N	89°18′04″O		X
CONK_F	*M. bijugatus*	Conkal	21°04′00″N	89°31′00″ O	X	
DZIT_F	*M. bijugatus*	Dzitas	20°50′20″N	88°31′42″W	X	
ESPI_F	*M. bijugatus*	Espita	21°00′46″N	88°18′17″O	X	
HALA_M	*M. bijugatus*	Halacho	20°29′00″N	90°05′00″O		X
KANT_F	*M. bijugatus*	Kantunil	20°47′45″N	89°02′05″O	X	
KOPO_M	*M. bijugatus*	Kopoma	20°38′52″N	89°53′55″O		X
MAN_M	*M. bijugatus*	Mani	20°23′11″N	89°23′25″O		X
MERI_M	*M. bijugatus*	Mérida	20°58′04″N	89°37′18″O		X
MOTU_F	*M. bijugatus*	Motul	21°05′42″N	89°16′59″O	X	
OXKU_ F	*M. bijugatus*	Oxkutzkab	20°18′10″N	89°25′06″O	X	
OXKU_M	*M. bijugatus*	Oxkutzkab	20°18′10″N	89°25′06″O		X
PIS_M	*M. bijugatus*	Piste	20°41′53″N	88°35′19″O		X
SUCI_F	*M. bijugatus*	Sucila	21°09′16″N	88°18′49″O	X	
TAME_F	*M. bijugatus*	Tahmek	20°52′27″N	89°15′22″O	X	
TEKI_M	*M. bijugatus*	Tekit	20°32′05″N	89°19′59″O		X
TELCH_F	*M. bijugatus*	Telchaquillo	20°38′45″N	89°27′51″O	X	
TICO_F	*M. bijugatus*	Ticopo	20°53′21″N	89°23′22″O	X	
MAXCA_F	*M. bijugatus*	Maxcanu	20°34′59″N	90°00′03″O	X	
ACAN_F	*M. oliviformis*	Acanceh	20°48′46″N	89°27′13″O	X	
MANI_F	*M. oliviformis*	Mani	20°23′11″N	89°23′25″O	X	
TELC_F	*M. oliviformis*	Telchaquillo	20°38′45″N	89°27′51″O	X	
SUCI_M	*M. oliviformis*	Sucila	21°09′16″N	88°18′49″O		X
DZIT_F	*M. oliviformis*	Dzitas	20°50′20″N	88°31′42″W		X

10-ng DNA in a 5 μL reaction using Titanium Taq DNA Polymerase (Clontech, Mountain View, CA). Electropherograms were analyzed using the software GeneMapper 4.0 (Applied Biosystems, Foster City, CA). Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) analysis, BLASTx was performed on the 31 microsatellite sequences.

Using the 31 polymorphic microsatellite markers before mentioned, genetic diversity was estimated in the 25 accessions of Spanish lime at the species and sex (female, male) levels using the average number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F), calculated with GenAlex version 6.5 (Peakall and Smouse 2012). Finally, to test the transferability of these 31 markers to Huaya India, allele sizes observed in the electropherograms were converted to presence or absence of alleles (1: presence; 0: absence) and data were analyzed by Neighbor Joining (N-J) (Nei 1972) and Principal Coordinate Analysis (PCoA) (Gower 1966) using NTSYSpc v.2.2 (Rohlf 2008).

Results and discussion

Sequencing and assembly of the microsatellite-enriched libraries of Spanish lime resulted in 9567

contigs (335 bp average length) containing repeats, a total of 3,203,629 nt. SSRFinder detected 10,117 SSRs on these contigs and the sequences were submitted to GenBank, NCBI with master accession number of KCYA00000000 and individual sequence numbers KCYA01000001-KCYA01009567. A total of 181 types of repeat motifs were found in microsatellite-enriched libraries of *M. bijugatus*, the most abundant are shown in Fig. 1. The count by motif length was: 20 motifs of 6 nt, 27 of 5 nt, 62 of 4 nt, 56 of 3 nt, 12 of 4 nt, and 4 of 1 nt. From the 10,117 repeats, Primer 3 designed 7551 high-stringency primer sets, from which 384 sets (Supplementary Table 1) were tested on four DNA accessions of Spanish lime: TARS 18206 and TARS 18219, 'Jose Pabon' (TARS 18218) and 'Melocoton' (TARS 18214). Results of the 384 primer sets tested using capillary electrophoresis are shown as allele sizes in Supplementary Table 2. In summary, 269 markers were monomorphic (70%), 106 polymorphic (28%), and nine (2%) did not amplify in one or more samples. BLAST analysis showed significant hits to two transcription factors (MB4380 and MB4221) and one asparagine-tRNA ligase (MB4221) (Table 3). Since markers MB4380 and MB4221 differentiate both species, polymorphism directly linked to proteins (BLASTx) could be associated to different biological functions. Whereas in the case of asparagine-tRNA ligase, this enzyme participates in alanine and aspartate metabolism (Davies and Marshall 1972).

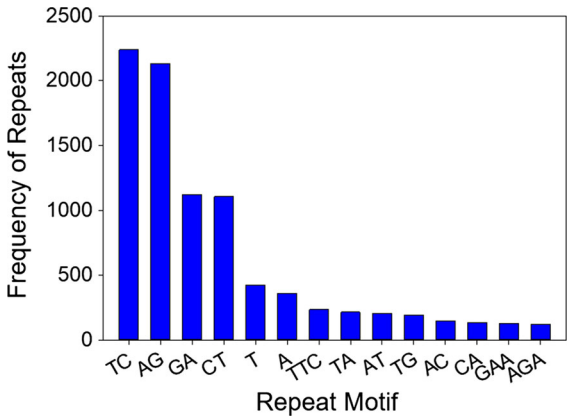

Fig. 1 Motifs and frequency of repeats detected in Spanish lime (*Melicoccus bijugatus*) microsatellite-enriched libraries

Spanish lime showed low genetic diversity at level species (A = 2.61, H_E = 0.38) (Table 4) when compared to that reported for lychee (A = 5.1, H_E = 0.53; Madhou et al. 2013). Also, we did not find differences in genetic diversity levels between male and female plants of Spanish lime. Factors that could explain our results are the relatively small sample size (25 accessions) used by us, or the existence of a founder effect as a result of the introduction of Spanish lime to Yucatan. There are no other publications on the genetic diversity of *Melicoccus*, and in general, there are limited studies on the genetic diversity of the family Sapindaceae using co-dominant markers such as microsatellites. Only one previous study had considered the genetic diversity of the genus *Melicoccus*, but that work was based on morphological and biochemical characteristics such as pulp pH and soluble solids, and was performed on cultivated accessions of Huaya India (Jiménez-Rojas et al. 2019).

Nine of the 31 markers tested showed unique alleles for Huaya India (Table 1), two markers (stv-MEB4366a; stv-MEB6464a) had only null alleles on the five Huaya India, and one marker (stv-MEB4008a) was monomorphic on the 30 accessions tested. The

Table 3 Results of BLASTx of DNA sequences of the microsatellites used in this study

Markers	Lowest E-value	Accession (E-value)	Description
MB2695	1.14E−46	XP_002314271	Hypothetical protein SORBIDRAFT, *Sorghum bicolor*
MB0695	4.83E−09	XP_002513909	Conserved hypothetical protein, *Ricinus communis*
MB1842	6.24E−15	XP_004290341	GATA transcription factor 5-like, *Fragaria vesca*
MB3227	7.77E−06	XP_002302663	Uncharacterized protein LOC7487284, *Populus trichocarpa*
MB4221	1.71E−19	XP_004249305	Asparagine-tRNA ligase cytoplasmic, *Solanum lycopersicum*
MB4380	2.67E−26	XP_002319143	NAC transcription factor 47, *Populus trichocarpa*
MB4947	2.25E−10	XP_004515508	Uncharacterized protein LOC101503933, *Cicer arietinum*
MB4008	3.23E−04	XP_002445301	Hypothetical protein SORBIDRAFT 07g008525, *Sorghum bicolor*

Table 4 Estimators of genetic diversity of 25 accessions of Spanish lime (*Melicoccus bijugatus*) cultivated in Yucatan, Mexico, using 31 microsatellite loci

Level	N	$A \pm$ SD	Ne $\pm$ SD	$H_O \pm$ SD	$H_E \pm$ SD	F $\pm$ SD
Species	25	2.61 $\pm$ 0.24	1.89 $\pm$ 0.12	0.39 $\pm$ 0.05	0.38 $\pm$ 0.04	0.03 $\pm$ 0.06
Female	15	2.52 $\pm$ 0.02	1.84 $\pm$ 0.13	0.39 $\pm$ 0.05	0.37 $\pm$ 0.04	0.05 $\pm$ 0.06
Male	10	2.36 $\pm$ 0.18	1.72 $\pm$ 0.10	0.38 $\pm$ 0.05	0.36 $\pm$ 0.04	0.08 $\pm$ 0.06

N, sample size; *A*, average number of alleles per locus; Ne, average number of effective alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F, fixation index; SD, standard deviation

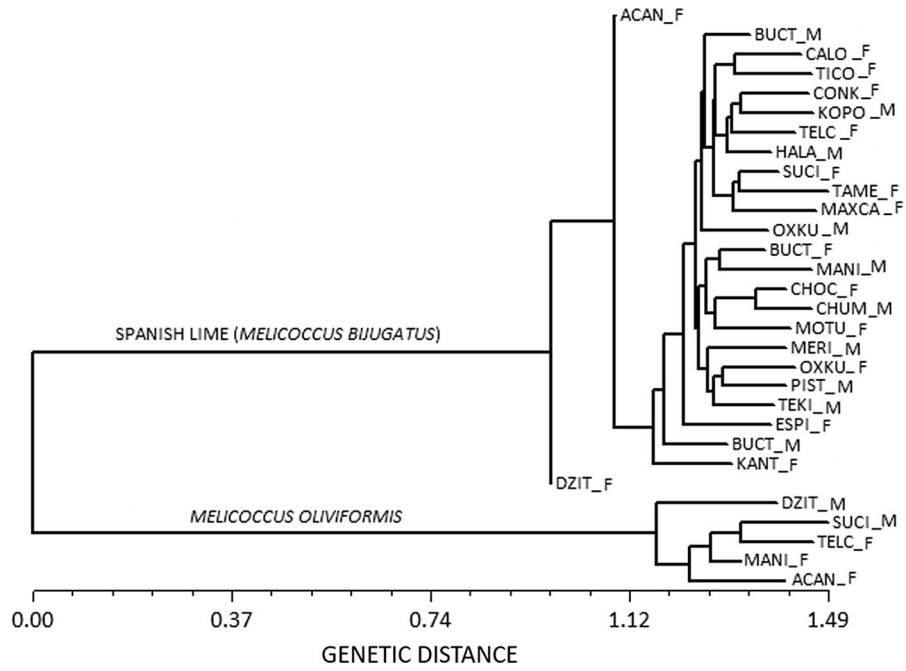

Fig. 2 Neighbor Joining analysis of 25 accessions of Spanish lime (*Melicoccus bijugatus*) and five accessions of *M. oliviformis* from Yucatan, Mexico, using 31 microsatellite loci. Accession names followed by _F: female; _M: male

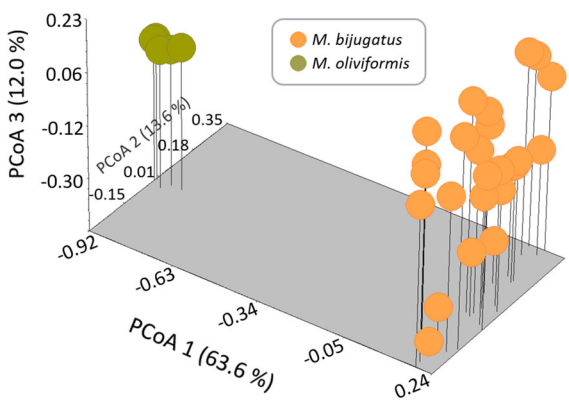

Fig. 3 Principal coordinate analysis (PCoA) of 25 accessions of Spanish lime (*Melicoccus bijugatus*) and five accessions of Huaya India (*M. oliviformis*) collected in the Yucatan peninsula, Mexico, using 31 SSR loci

N-J (Fig. 2) and the PCoA (Fig. 3) analyses clearly discriminated between Spanish lime and Huaya India, but not between male and female plants of Spanish lime. The accessions of Spanish lime did not indicate association to their geographic origin, maybe because this species is not native of Mexico (Martin et al. 1987; Acevedo-Rodríguez 2003). A similar pattern was observed in *M. oliviformis*, a species native of Mexico, maybe for the low number (5) of individuals included in this study.

Conclusions

Microsatellite markers developed here showed high-quality amplification in Spanish lime and

transferability to Huaya India. These markers could potentially be expanded to the other species of *Meliccocus* and even *Talisia*, a closely genus native of the Amazon with 52 species (Acevedo-Rodríguez 2003). Limited sample collections of *Melicoccus* and *Talisia* species indicate they could be at risk of extinction, including *M. jimenezzi*, a Dominican Republic endemic restricted to a small population (Acevedo-Rodríguez 2003). There are limited genetic diversity studies in Sapindaceae, and none in *Melicoccus*. This prevents knowing the state of conservation of *Melicoccus* and how this information could impact the preservation of genetic resources that are vital for people in the tropics. The markers reported here will help generate information in relation to conservation genetics, improvement of elite cultivars and breeding programs.

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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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