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Short Communication

Development and characterization of microsatellite markers for two subspecies of *Handroanthus chrysanthus*

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Abstract

An understanding of the genetic diversity and structure of plant species is essential in order to comprehend the degree of biodiversity loss and to develop successful restoration programs. *Handroanthus* is an important genus that presents one of the most valuable timbers of South America. *Handroanthus chrysanthus* is an important species distributed in Central and South America. Microsatellite markers are not previously developed for this species. Ten microsatellites for *Handroanthus chrysanthus* developed using high-throughput sequencing are presented here. The usefulness of these microsatellite loci for the genetic analysis of subspecies *H. chrysanthus* subsp. *chrysanthus* (distributed in coastal dry forests) and subspecies *H. chrysanthus* subsp. *meridionalis* (distributed in premontane moist forests) is analyzed. At least eight polymorphic microsatellites are useful for each subspecies, seven of which can be used in both subspecies.

Key words: dry forest, Ecuador, Guayacán, *Handroanthus chrysanthus*, microsatellites, premontane forest, subspecies.

Resumen

Comprender de la diversidad y estructura genética genética de las especies vegetales es esencial para entender el grado de pérdida de la biodiversidad y para desarrollar programas de restauración exitosos. *Handroanthus* es un género importante que presenta una de las maderas más valiosas de Sudamérica. *Handroanthus chrysanthus* es una especie importante, distribuida en América Central y del Sur. Sin embargo, no se han desarrollado previamente marcadores de microsatélites para esta especie. En este trabajo presentamos diez marcadores microsatellites desarrollados para *Handroanthus chrysanthus* mediante secuenciación de alto rendimiento. Se analiza la utilidad de estos loci microsatélites para el análisis genético de la subespecie *H. chrysanthus* subsp. *chrysanthus* (distribuida en bosques secos) y la subespecie *H. chrysanthus* subsp. *meridionalis* (distribuida en bosques húmedos premontanos). Por lo menos ocho microsatélites polimórficos son útiles para cada subespecie, siete de los cuales pueden utilizarse en ambas subespecies.

Palabras clave: bosque seco, Ecuador, Guayacán, *Handroanthus chrysanthus*, microsatélites, bosque premontano, subespecie.

Knowledge about species biodiversity is increasing in Ecuador (ter Steege *et al.* 2019), but knowledge about genetic diversity at the population level is still very poor. Such information is important in order to fight habitat loss and deforestation, the main pressures that affect Southern Ecuador (Tapia-Armijos *et al.* 2015) and cause species extinctions (Ehrlich & Pringle 2009). Species extinction is the final step in the long process of population decline (Ceballos *et al.* 2017) and decreasing

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genetic diversity. An understanding of genetic population structure and diversity is also crucial for the establishment of successful restoration projects, and can mean the difference between achieving or not achieving project goals, including long term plant persistence and restoration of functioning ecosystems (Kettenring *et al.* 2014).

Handroanthus Mattos (Bignoniaceae) is a genus that comprises 30 species distributed throughout Central and South America with one species in the Antilles (Grose & Olmstead 2007). *Handroanthus chrysanthus* (Jacq.) S. Grose is an emblematic species of the genus with a broad distribution in Central and South America. Although the species is not considered on the IUCN red list, the three subspecies described for *H. chrysanthus* are widely distributed in Ecuador (Jørgensen & León-Yánez 1999) in regions with a highest pressure caused by deforestation (Rahbek *et al.* 2019; Tapia-Armijos *et al.* 2015; Manchego *et al.* 2017). Moreover, the species have one of the more valuable timber of South America, and its use caused overexploitation and subsequent threat (Schulze *et al.* 2008).

Microsatellite loci primers to study population diversity in *Handroanthus* has been developed for *H. billbergii* (Bur & K. Schum) S. Gorse (Morillo *et al.* 2016). In that study, microsatellite loci primers were also tested on *H. chrysanthus* with a high rate of amplification; however, they did not report which subspecies was used for cross-amplification assays.

In this work we describe 10 microsatellite loci that have been developed and proved for two *H. chrysanthus* subspecies with different distribution patterns. *Handroanthus chrysanthus* subsp. *chrysanthus* (Jacq.) S. Grose is present in the coastal dry forest below 500 m and *H. chrysanthus* subsp. *meridionalis* (A. H. Gentry) S. Grose is distributed in the premontane moist forest at 1,200–2,000 m (Patzelt 1996). The timber of both subspecies is used in construction and subsp. *chrysanthus* is also used in agroforestry projects (De la Torre *et al.* 2008).

The genomic DNA from two specimens, one from the dry forest and one from the premontane moist forests population was isolated from 20 mg of dry leaf material using the protocol from Curto *et al.* (2013). Libraries prepared as described in Deck *et al.* (2016) were sequenced in a 300 bp paired-end sequencing run on an Illumina MiSeq system (Illumina, USA). This was done on the genomic service unit from the Ludwig Maximillian University of Munich. The quality of the resulting reads was evaluated with FASTQC (Andrews 2010) and low-quality regions and adapter sequences were trimmed out with cutadapt (Martin 2011). This was done with a 20 bp sliding window starting at the 3' end excluding regions with an average quality below 20. The Illumina TrueSeq adapter sequences were used to find potential adapters in the reads using cutadpat default settings. Paired reads showing a significant 15 bp overlap were merged using PEAR (Zhang *et al.* 2014). After quality control 3533155 and 524591 reads were kept, to each library respectively, and used for SSR search. 2672 and 1860 sequences contained microsatellite motifs and were used for primer design. The screened for di-, tri- and tetra-nucleotide microsatellite motifs using MSATCOMMANDER program (Faircloth 2008). PRIMER3Web was used (Untergasser *et al.* 2012) to search locus-specific primers considering PCR products within a size range between 100 and 300 bp, optimal melting temperature between 58–62 °C and GC content between 40 and 60%. One primer per locus was tagged with a M13 universal sequence on its 5'end (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569636.v1>) to use the dye-labeling method (Godinho *et al.* 2011). The OLIGO-ANALYZER 3.1 (Integrated DNA Technologies) was used to estimate the presence of self-dimers, heterodimers and hairpins among the primers.

According to Koskinen *et al.* (2004), Vartia *et al.* (2014) and Arthofer *et al.* (2018) 8 to 20 loci are enough to detect population structure. A total of 28 primers were designed. These primers were initially tested in samples of the two subspecies (five samples per subspecies). These PCRs (Polymerase Chain Reactions) were performed in a final volume of 20 mL, containing 1X PCR buffer, 0.1 mM of each dNTP, 0.2 mM of each primer, 1.5 mM $MgCl_2$, 0.2 unit of Go TAQ DNA polymerase (Promega) and 30–50 ng of DNA template. The products were amplified with the following conditions: one cycle at 94 °C for 3 min, 35 cycles at 94 °C for 30 sec, annealing temperature according to each primer pair for 45 sec and 72 °C for 1 min, and one cycle at 72 °C for 10 min. PCR amplification was verified by electrophoresis in a 2% agarose gel. Ten primers that amplified well and presented evidence of polymorphism were selected to standardize posterior multiplex PCRs.

Two multiplex PCRs were used for a total of 107 samples, 47 specimens from two populations:

(Arenillas and Mangahurco) of *H. chrysanthus* subsp. *chrysanthus* using 8 loci and 60 specimens from two populations (Chitoque and Valladolid) of *H. chrysanthus* subsp. *meridionalis* using 9 loci (Tab. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569636.v1>). Vouchers of both subspecies are placed at the Herbarium of Universidad Técnica Particular de Loja (HUTPL14150 - HUTPL14255). The four populations are located in Southern Ecuador (Fig. 1).

Both PCRs were performed in a final volume of 7 mL containing 1X QIAGEN multiplex master mix and 10–60 ng of genomic DNA. The first multiplex grouped primers: *Hchrys2-F* (0.006 mM), *Hchrys2-R* (0.06 mM), *Hchrys9-F* (0.007 mM), *Hchrys9-R* (0.07 mM), *Hchrys12-F* (0.006 mM), *Hchrys12-R* (0.06 mM), *Hchrys16-F* (0.01 mM), *Hchrys16-R* (0.1 mM), *Hchrys25-F* (0.009 mM), *Hchrys25-R* (0.09 mM), *Hchrys26-F* (0.014 mM), *Hchrys26-R* (0.14 mM), *6-FAM-M13* (0.09 mM), *PET-M13* (0.13 mM), *NED-M13* (0.07 mM) and *VIC-M13* (0.06 mM). The second multiplex grouped primers: *Hchrys4-F* (0.009 mM), *Hchrys4-R* (0.09 mM), *Hchrys6-F* (0.0043 mM), *Hchrys6-R* (0.043 mM), *Hchrys15-F* (0.009 mM), *Hchrys15-R* (0.09 mM), *Hchrys28-F* (0.01 mM), *Hchrys28-R* (0.1 mM), *6-FAM-M13* (0.09 mM), *PET-M13* (0.09 mM), *NED-M13* (0.043 mM) and *VIC-M13* (0.1 mM). Both PCRs were amplified using a touchdown PCR procedure: initial denaturation at 95 °C for 15 min, 13 cycles of 94 °C for 30 sec, 60 °C for 50 sec (with a decrease of 0.5 °C each cycle), 72 °C for 1 min, 22 cycles of 94 °C for 30 sec, 54 °C for 50 sec, 72 °C for 1 min and a final extension at 72 °C for 10 min.

For fragment separation 1 mL of the PCR product was mixed with 10 mL of Hi-Di Formamide and 0.15 mL of GeneScan LIZ 600 size standard and analyzed on a 3500 Genetic Analyzer (Applied biosystem, USA) according to manufacturer's instructions and recorded in GeneMapper version 4.1 software (Applied biosystem).

The Number of alleles (A), observed heterozygosity (Ho) and expected heterozygosity (He) for each locus and population were analyzed with GenAlEx 6.50 (Peakall & Smouse 2012). The exact Hardy-Weinberg global test of heterozygosity deficiency and linkage disequilibrium was

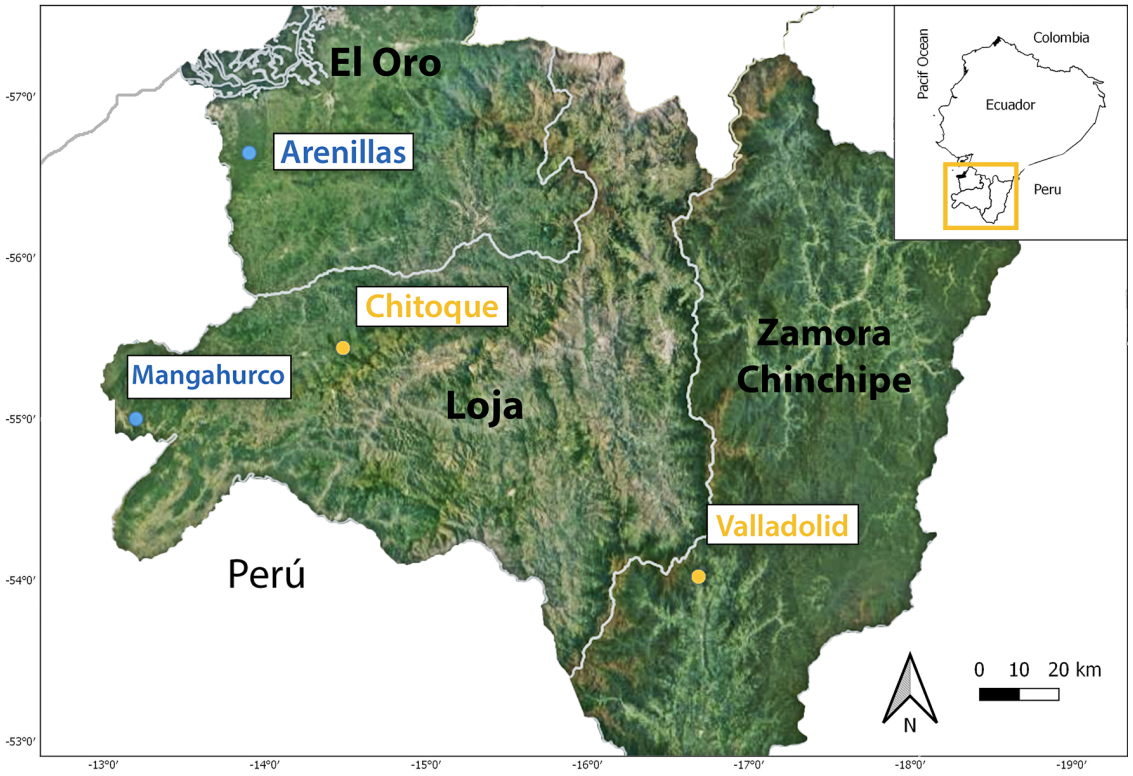

Figure 1 – Location of the populations of *Handroanthus chrysanthus* subsp. *chrysanthus* (Arenillas and Mangahurco) and *Handroanthus chrysanthus* subsp. *meridionalis* (Chitoque and Valladolid).

calculated in GENEPOP on the Web (Raymond & Rousset 1995; Rousset 2008). Parentage analysis was performed using *Cervus* program (Slate *et al.* 2000). Null allele frequencies were calculated using FreeNA (Chapuis & Estoup 2007; Chapuis *et al.* 2008).

A total of 28 microsatellites were tested from which 10 amplified well and presented evidence of polymorphism when evaluated by 2% agarose gel electrophoresis. Each multiplex genotyping PCR was repeated at least three times. If one locus failed to amplify in some samples we tried the amplification of this single locus to be sure that it was not a result of PCR problems. The amplification success and null allele frequencies were different for each subspecies (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569636.v1>). For *H. chrysanthus* subsp. *chrysanthus* two loci (*Hchry16* and *Hchrys26)* did not amplify in more than 50% of the samples, and locus *Hchrys16* also had a null allele frequency higher than 10%. For *H. chrysanthus* subsp. *meridionalis* a different locus (*Hchrys4*) failed to amplified in almost 100% of the samples. For this locus the null allele frequency was high (more than 30%). A total of 90 and 92 alleles were scored for subsp. c*hrysanthus* and subsp. *meridionalis*, respectively (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569636.v1>).

We genotyped a total of 107 specimens from two subspecies (Tab. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569636.v1>). Loci that had a high percentage of failed amplification were not considered for each subspecies. Any of the 10 loci exhibit linkage disequilibrium. When parentage analysis was performed we did not find any parent offspring relationship. A good probability of identity (0.0001 or less) was achieved using 6 loci for subsp. *chrysanthus* and 5 for subsp. *meridionalis* so that even if the less polymorphic loci are not considered in future studies a good probability of identity can still be achieved. High overall observed heterozygosity (0.524 and 0.542) and expected heterozygosity (0.518 and 0.541) were found in the screened populations of subsp. *chrysanthus*. For subspecies *meridionalis,* similar values were found (Ho: 0.524 and 0.428, He: 0.556 and 0.564) except for the observed heterozygosity in the Valladolid population. The exact test of heterozygosity deficiency showed different levels of significance in the two subspecies. In subsp. *meridionalis* 6 of the 9 loci have significant values in the Valladolid population, but only one locus deviated from Hardy-Weinberg equilibrium in the Chitoque population. This could be more related to the specific characteristics of the populations rather than to issues with specific loci in this subspecies. Seven microsatellite loci are common to both subspecies.

We presented the characterization of ten microsatellites which can be used for the analysis of *H. chrysanthus* subsp. *chrysanthus* and *meridionalis* and potentially for *H. chrysanthus* subsp. *pluvicola.* The microsatellites can be used in future studies of population diversity and structure of this species. Our results confirm the necessity to verify the applicability of specific microsatellite loci when intraspecific genetic variation is present.

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