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Morphometric and molecular differences among *Calvertius tuberosus* (Coleoptera: Curculionidae) populations associated with Andean and coastal populations of *Araucaria araucana* in the La Araucanía Region, Chile

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ABSTRACT

Calvertius tuberosus (Curculionidae) lives exclusively on *Araucaria araucana* trees (commonly known as pehuen) in southern Chile. In this study, morphometric and molecular genetic analyses of Andean and coastal populations of *C. tuberosus* were performed to evaluate evolutionary divergence associated with the discontinuity of the *Araucaria* forest between the coastal and Andean regions. Specimens of *C. tuberosus* were collected in Nahuelbuta National Park, Villa Las Araucarias, and Malalcahuello National Reserve and were classified and stored at the Animal Biotechnology Researching Laboratory (LINBA), University of La Frontera, Temuco, Chile. Thirteen morphometric parameters and the expression patterns of ISSR (inter-simple sequence repeat) markers were analyzed. Morphometric data revealed high phenotypic similarity between coastal populations. The genetic analysis revealed a high similarity between coastal populations (genetic identity, 93%), which were differentiated from the Andean population (genetic identity, 84%). This study contributes new genotypic and phenotypic data for the *C. tuberosus* populations in forest ecosystems of *A. araucana*, and clarifies the associations between these characteristics and the geographic distributions of populations.

Introduction

In Chile, there are over 4200 species of Coleoptera belonging to 97 families (Elgueta, 2008), representing 30% of all insects in Chile, with little diversification in most genera (Vergara et al., 2006) and high levels of endemism at the species level. Many of these genera are shared with those found in Australia and New Zealand, instead of South American tropical zones (Arias, 2000). Curculionidae is a diverse family, with almost 4600 genera and 51,000 species (Oberprieler et al., 2007).

Calvertius tuberosus (Fairmaire & Germain, 1860) (Coleoptera: Curculionidae) is found between the Biobío (36°46′22″S, 73°03′47″W) and La Araucanía (38°54′00″S, 72°42′00″W) regions in southern Chile (Arias, 2000), and is exclusively found on *Araucaria araucana* ((Mol) Koch, 1869) (Pinales: Araucariaceae) trees, with a frequency of almost 30% (Elgueta et al., 2008).

C. tuberosus is the largest among the 23 species of curculionids and other related families, on this host (Kuschel, 2000). Adults walk on the trunk or feed on leaves and soft shoots, and larvae are found in the subcortical zone in fallen or standing trees, where they feed on phloem, although they frequently become xylophages at the end of their development (Barriga et al., 1993; Morrone, 1997; Kuschel, 2000; Elgueta and Marvaldi, 2006). Morrone (1997) indicated that this beetle is a secondary invader that does not attack healthy trees, but enters branches previously affected by bark beetles (Scolytinae).

Araucaria araucana is an endemic species in South American temperate forests along the Andes mountains from 37°27′S to 40°03′S (Moreno et al., 2011). Approximately 97% of its populations form extensive pure forests, often on steep volcanic hills, and

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are associated with temperate rainforest species (Hechenleitner et al., 2005).

Some relatively small and disjunct populations occur in the Nahuelbuta mountains at the Nahuelbuta National Park (33°37′00″S, 79°02′00″W) and surrounding areas, including Villa Las Araucarias (38°00′17″S, 72°57′56″W). In this latter area, *Araucaria* are found in highly altered environments dominated by mixed forest of *Nothofagus* spp. (Fagales: Nothofagaceae) and exotic trees, such as *Eucalyptus globulus* (Labill) and *Pinus radiata* (D. Don).

The remaining *Araucaria* forests belong to private owners and are permanently subject to high levels of disturbance by the inadequate extraction of their edible fruit, fires, logging, and substitution with commercial forest plantations (Donoso et al., 2006). The ecosystems of both areas are characterized by a moist Mediterranean climate with differences related to altitude and exposure; their soils are composed of metamorphic materials and, in some locations, granite (Donoso et al., 2008). It has been proposed that geographical isolation between coastal and Andean *A. araucana* populations results in genetic population differentiation (Raffi and Dodd, 1998).

Morphometry was among the first methods used in biodiversity and phylogenetic studies and is still applied, despite the wide range of molecular techniques used currently (Wanek and Sturmbauer, 2015). Morphometric analyses are used in taxonomy, but are also used in coevolution and phylogenetic studies of diverse groups of insects, such as aphids, bees, grasshoppers, and beetles (Sánchez-Ruiz and San Martín, 2000). Morphometric measurements are widely used in approaches that integrate systematics with molecular data and can lead to taxonomic revisions, comparable to phylogenies created from DNA. When correctly selected, morphometric parameters can be used to establish phylogenetic relationships, especially for species that are not easy to distinguish owing to a lack of diagnostic characters (Przybycien and Waclawik, 2015).

At the genetic level, nucleotide sequence differences can be used to study evolutionary relationships among species. For instance, Woese and Fox (1977) classified prokaryotes based on ribosomal genes. Phylogenies created from DNA sequences can provide useful insight into the evolutionary history of genes and organisms (Yang and Rannala, 2012). During evolution, genetic material accumulates mutations that potentially result in phenotypic changes (Olsen and Woese, 1993).

Inter-simple sequence repeats (ISSRs) are a sensitive genetic marker for studies of polymorphism (Bornet and Branchard, 2004) within populations based on the absence or presence of a genomic element and the length of the amplified intermediary sequence (Zietkiewicz et al., 1994). ISSRs in the genomes of plants and animals are highly variable and therefore are commonly used in population genetic studies (Tikunov et al., 2003). ISSR analyses do not require high concentrations of DNA, and primer development does not require previous knowledge of the genome sequence of the organism under study (Joshi et al., 2000). The high degree of polymorphism and wide distribution of microsatellites enable the detection of low levels of differentiation (Yua, 2011).

Other molecular tools have been used to characterize *A. araucana* populations. For example, Marchelli et al. (2010) studied their possible pre-Pleistocene origin using chloroplast and mitochondrial DNA sequences. Based on nuclear and mitochondrial gene sequence analyses, Sequeira and Farrell (2001) investigated the phylogenetic relationships and the estimated divergence times of bark beetles associated with *Araucaria* in Australia and South America. The structure and genetic diversity of populations in South America have been studied based on the composition of foliar epicuticular wax alkanes (Rafii and Dodd, 1998), RAPD markers (Bekessy et al., 2002), nuclear microsatellites (Martín et al., 2014), and AFLPs (Marconi et al., 2011).

In this study, the morphological and genetic characteristics of *C. tuberosus* specimens from coastal populations (Nahuelbuta National Park, hereafter Nahuelbuta, and Villa Las Araucarias) and the Andean National Reserve (Malalcahuello National Reserve, hereafter Malalcahuello) of the La Araucanía region, Chile were evaluated with respect to differences among *A. araucana* populations.

Materials and methods

Populations

C. tuberosus were collected from the bark of fallen and standing *A. araucana* threes in three areas in the la Araucanía region, i.e., Malalcahuello (38°24′21.56″S, 71°35′46.08″W), Villa Las Araucarias (38°29′12.65″S, 73°15′41.13″W), and the Nahuelbuta National Park (37°47′33.69″S, 72°59′53.36″W).

Morphometric measurements

Forty specimens from Villa Las Araucarias (27 male, 13 female), Nahuelbuta (30 male, 10 female), and Malalcahuello (32 male, 8 female) were included in the analyses. For each specimen, 13 parameters were measured under a Leica EZ4 stereoscopic magnifier (Wetzlar, Germany). The morphometric parameters were as follows: prothorax length, prothorax base width, prothorax maximum width, elytra length, elytra base width, elytra maximum width, elytra minimum width, elytra apex width, pedicel length, flagellum length, rostrum length, rostrum apex width, and rostrum base width (Fig. 1). Additionally, coloration was recorded for specimens in each population. These morphometric measurements were used to create a dendrogram using Nei's (1972) model implemented in PAST 3.14 (Hammer et al., 2001). Q2

Total DNA extraction from C. tuberosus

Individuals were stored at −80 °C at the Animal Biotechnology Research Laboratory (LINBA), University of La Frontera, Temuco, Chile, after grinding each specimen in a China mortar following treatment for 10 min with UV light, yielding 1–2 mg of homogenized tissue from each insect. Samples were processed using the AxyPrep Multisource Genomic DNA Miniprep Extraction Kit (Axygen Biosciences, Tewksbury, MA, USA).

Design of ISSR markers

Seventeen ISSR primers (Table 1) were selected based on the methods of Korpelainen et al. (2007). Seven primers (AC-T, CA-G, GA-C, AG-C, AC-C, CA-A, CAG) were prepared at the Animal Biotechnology Researching Laboratory (LINBA) for PCR amplification with purified DNA of *C. tuberosus*, following the methods of Pérez de la Torre (2012), with modifications. The amplification conditions were as follows: 10 min of denaturation at 95 °C, 40 s at 90 °C (45 cycles), 45 s of reheating at 50 °C (45 cycles), 90 s of initial extension at 72 °C (45 cycles), 10 min of final extension at 72 °C. The reaction mixture contained the following: 10 μL of Maxima SYBR Green qPCR Master Mix (2×), 1 μL of DNA (200 ng), 1 μL of 17 ISSR primers, and 8 μL of H_2O ultra-pure (20-μL final volume). The amplification reaction was performed using a MultiGene Gradient Thermocycler (Labnet International Inc., Edison, NJ, USA).

PCR amplification of ISSR markers

Of the 17 primers evaluated according to their patterns of polymorphism, five were selected ($[AC]_8$-T, $[GA]_9$-T, $[GA]_8$-C, $[GA]_9$-A,

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