Revista Brasileira de Entomologia 62 (2018) 119–124

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA
FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution

www.rbentomologia.com

Systematics, Morphology and Biogeography

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

Luis Huala Jimenez[a], Ramón Rebolledo Ranz[b], Rubén Carrillo López[b], Mario Elgueta[c], Marco Paredes Honorato[a,*]

[a] *Universidad de La Frontera, Departamento de Ciencias básicas, Temuco, Chile*
[b] *Universidad de La Frontera, Departamento de Ciencias Agronomicas y Recursos Naturales, Temuco, Chile*
[c] *Museo de Historia Natural, Área de Entomología, Santiago, Chile*

ARTICLE INFO

Article history:
Received 25 September 2017
Accepted 24 December 2017
Available online 2 February 2018
Associate Editor: Adriana Marvaldi

Keywords:
Araucaria forests
ISSR markers
Morphometry

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

* Corresponding author.
E-mail: marco.parades@ufrontera.cl (M.P. Honorato).

https://doi.org/10.1016/j.rbe.2017.12.004

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).

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,

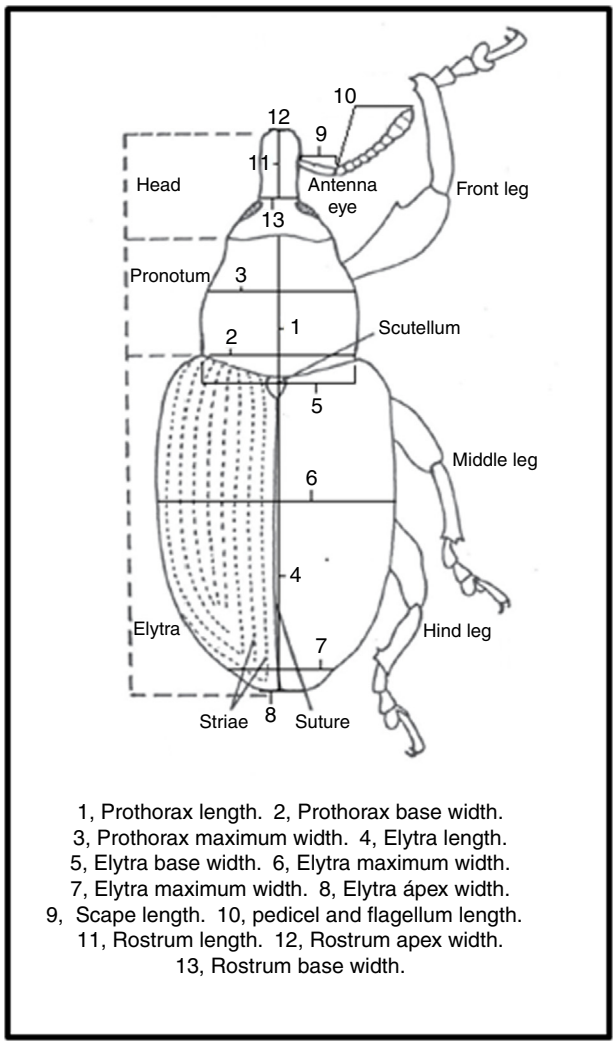

Fig. 1. Morphology of a generalized Curculionidae, with the morphometrics measurements used (adapted from Marvaldi and Lanteri, 2005).

Table 1
Names and sequences of ISSR primers (Korpelainen et al., 2007).

Names	Sequences	Names	Sequences
AC-T	$[AC]_8T$	CAA	$[CAA]_5$
CA-G	$[CA]_9G$	CA-A	$[CA]_9A$
GA-T	$[GA]_9T$	CAG	$[CAG]_6$
GA-C	$[GA]_8C$	ATG	$[ATG]_5$
GA-A	$[GA]_9A$	GA-C	$[GA]_9C$
AG-T	$[AG]_8T$	AC-G	$[AC]_9G$
AG-C	$[AG]_8C$	CA-T	$[CA]_9T$
AG-G	$[AG]_9G$	GA-C	$[GAC]_5A$
AC-C	$[AC]_8C$		

and $[CA]_9$-G). The PCR conditions were as follows: 10 μL of Maxima SYBR Green qPCR Master Mix (2×), 1 μL of DNA, 1 μL of each ISSR primer, and 8 μL of ultra-pure H_2O (final volume, 20 μL). The DNA amplification procedure was performed using a MultiGene Gradient Thermocycler (Fig. 2).

In the analysis of ISSR expression patterns, only polymorphic and reproducible bands were considered, and values of 1 or 0 were assigned to indicate presence or absence. Data were classified in a binary matrix and used to estimate genetic distances based on Nei, 1972 model and these distances were used to build a dendrogram by the UPGMA method (Sneath and Sokal, 1975) with POPGEN 1.32 (Yeh et al., 1999).

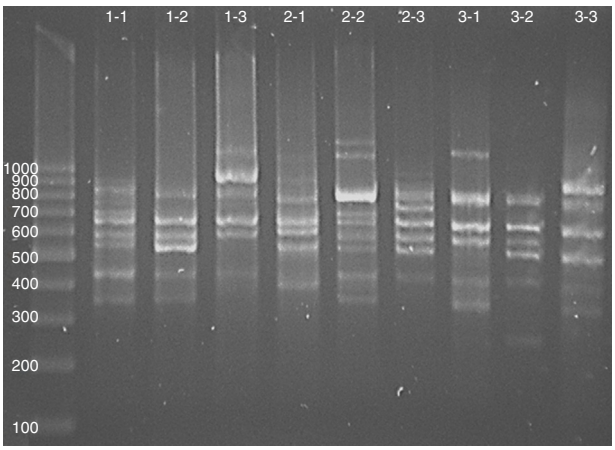

Fig. 2. ISSR patterns of expression for *C. tuberosus* from Malalcahuello (1-1, 1-2, 1-3), Villa Las Araucarias (2-1, 2-2, 2-3) and Nahuelbuta (3-1, 3-2, 3-3).

Results

A dendrogram was generated based on ISSR-derived genetic distances, reflecting the morphometric relationships among specimens obtained from three populations of *C. tuberosus*. There was greater morphological similarity between the specimens obtained from Villa Las Araucarias and Nahuelbuta (both coastal mountain locations) than between coastal and Andean populations. Moreover, the specimens obtained from Malalcahuello exhibited similar morphological features to those of the specimens obtained from Villa Las Araucarias (Fig. 3).

The specimens obtained from the Villa Las Araucarias and Nahuelbuta populations presented a marked black or dark brown color, while the Malalcahuello specimens exhibited a deep red color.

In the ISSR analysis, 45 bands were detected, of which 43 (96%) were polymorphic, with a size of 250–1100 bp (Fig. 2). The coastal populations (Nahuelbuta and Villa Las Araucarias) exhibited a genetic identity of 93%. The Malalcahuello population (Andes Mountains) had genetic identities of 84% and 87% in comparisons with the Villa Las Araucarias and Nahuelbuta populations, respectively (Fig. 4).

A genetic distance value of less than 0.07 was observed between the coastal populations. The genetic distances between the Andean population (Malalcahuello) and the Villa Las Araucarias and Nahuelbuta populations were 0.17 and 0.14, respectively (Fig. 4).

Discussion

Differences in body size between males and females are common in insects; typically, females are larger than males (Posadas et al., 2007). In Curculionidae, sexual dimorphism is common in the rostrum, i.e., the female rostrum is generally larger and flatter than that of the male (Soto and Reyes, 2014). Nevertheless, in *C. tuberosus*, rostrum size did not differ substantially between females and males; the observed morphometric variation could be the result of geographic isolation among *C. tuberosus* populations.

The morphometric analysis indicated the presence of geographic variation; in particular, the coastal populations (Villa Las Araucarias and Nahuelbuta) exhibited similar morphological patterns, which differed from those of the Andean population (Malalcahuello) (Fig. 3). The morphological and genetic similarity of coastal populations of *C. tuberosus* could be attributed to a more recent biogeographic separation of these populations.

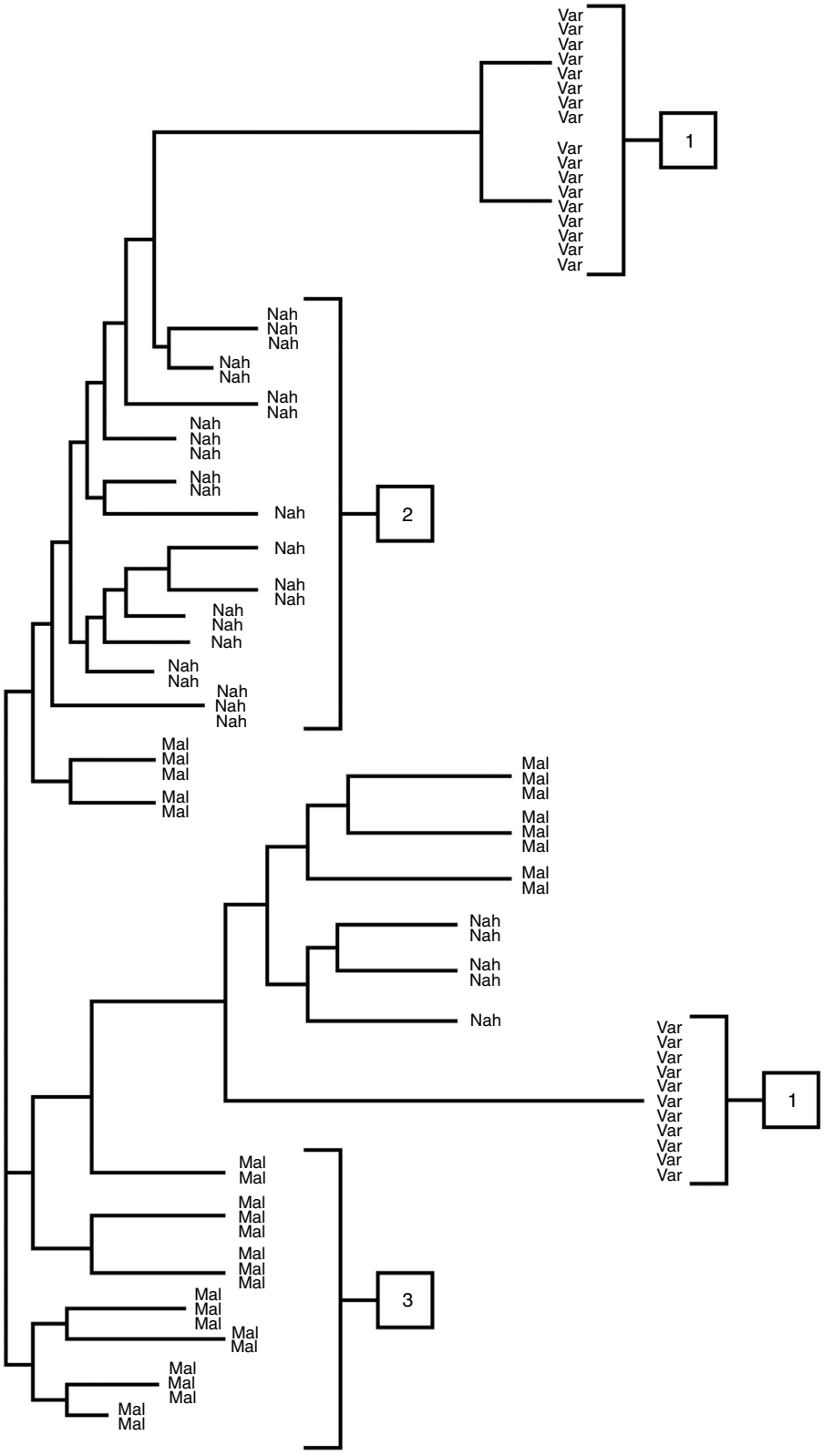

Fig. 3. Neighbor-joining dendrogram of morphometrics measurements; 1. Villa Las Araucarias, 2. Nahuelbuta, 3. Malalcahuello.

The morphological and genetic characteristics of the coastal and Andean populations of *C. tuberosus* could be a consequence of the geographical separation their host, *A. araucana*, which has been affected by large-scale environmental changes. For example, since Pleistocene glaciation events, the tree persisted in small populations during the Last Glacial Maximum (LGM) in the coastal range of Chile and some areas of the Andes Mountains (Villagrán, 2001; Martín et al., 2014), with a discontinuous distribution. The

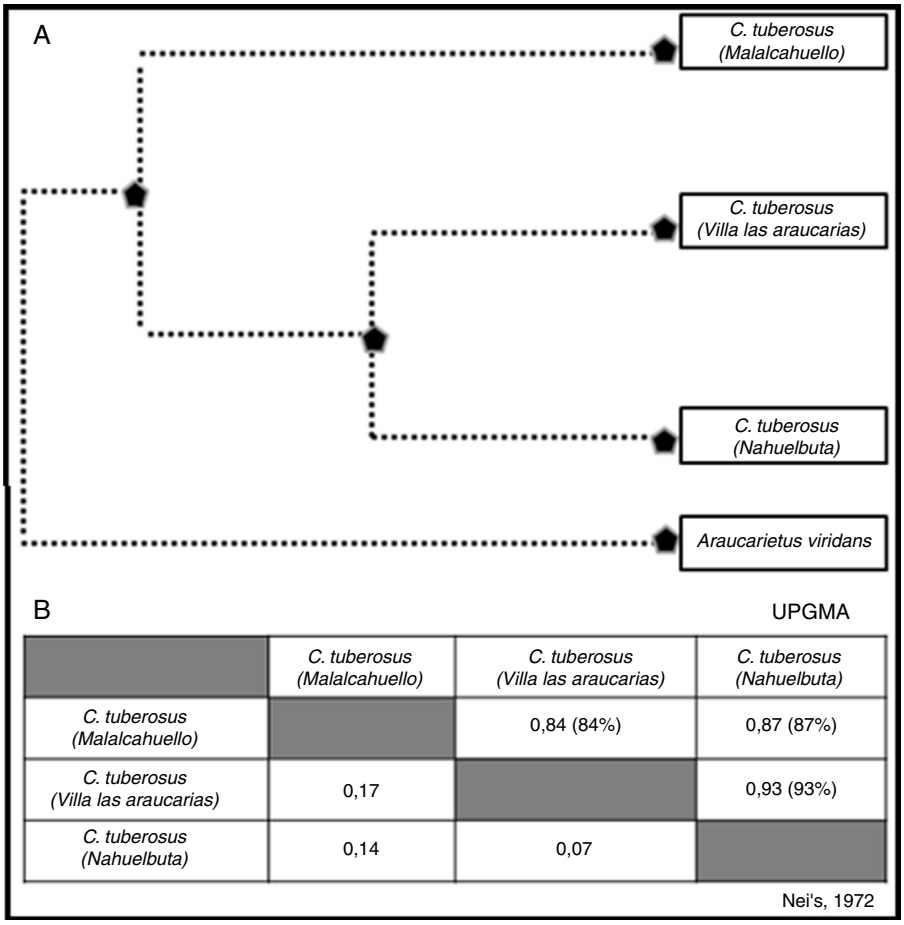

Fig. 4. ISSR analysis. (A): Dendrogram for the populations studied (control group *A. viridans*).

substantial distance between populations suggests the possibility of population genetic changes, such as inbreeding, gene drift, and altered gene flow (Marchelli et al., 2010).

More recent catastrophic events, such as the Villarrica, Llaima, Lonquimay, and Copahue volcanic eruptions (Heusser et al., 1988) and serious deforestation over the last century, have resulted in the degradation or replacement of about 120,000 ha of native forest (Herrmann, 2006), leading to further differences between the Andean and coastal populations by a lack of a vegetative continuum, limiting insect dispersal.

Despite a close relationship between the populations of Villa Las Araucarias and Nahuelbuta, there were clear genetic and morphological differences between the specimens of *C. tuberosus* in the populations. Current data indicate that anthropic intervention at Cordillera de Nahuelbuta (the mountain range that connects both populations) has resulted in over 70% loss of native vegetation (Wolodarsky-Franke and Díaz, 2011) and produced habitat fragmentation and more substantial population isolation.

Differentiation in morphometric parameters between geographically separated populations explains, to some extent, evolutionary processes and is considered the first stage in allopatric speciation (Olivero et al., 2012). In *C. tuberosus*, the interruption of gene flow between populations in diverse historical periods could have resulted in reproductive isolation, which would eventually lead to genetic differentiation and evolutionary divergence.

The genetic differences and similarities among populations of *C. tuberosus* (Fig. 4) can be compared with genetic variation in its obligate host, *A. araucana*. These comparative analyses allow us to infer that changes in the geographic distribution of *Araucaria* influence the observed variation in both species, thereby explaining genetic variability in coastal and Andean populations (Bekessy et al., 2002).

The ISSR markers revealed a more distant relationship (based on distance and genetic identity) between the coastal populations (Nahuelbuta and Villa Las Araucarias) and the Andean population (Malalcahuello) than between the two coastal populations (Fig. 4). These results are consistent with previous analyses of *A. araucana* Andean and coastal populations indicating significant genetic differences using RAPDs (Bekessy et al., 2002) and microsatellites (Martín et al., 2014). Similar differences between *Araucaria* populations have been described by Rafii and Dodd (1997) based on the proportional composition of foliar epicuticular wax alkanes.

It is not possible to understand the natural world without extensive knowledge of the morphological characteristics of organisms, which play central roles in life cycles, geographical distributions, identification, conservation, evolution, development, and delimitation of species (Wortley and Scotland, 2006). The inclusion of morphological data substantially improves the results of molecular phylogenetic analyses, proving to be useful not only for the resolution of taxonomic problems, but also for coevolution studies and phylogenetic inferences (Przybycien and Waclawik, 2015). The linkage of morphometric and molecular analyses is powerful for studies of population structure and the adaptive significance of trait divergence (Lee and Lin, 2012), to identify biotypes of the same species on different hosts (Fekrat et al., 2014), and for the geographic delimitation of species (Schwarzfeld and Sperling, 2014). Therefore, integrative analyses including morphometric measurements and DNA sequences are needed for a comprehensive understanding of the autoecology of species.

Conclusions

This study contributes new genotypic and phenotypic data for the *C. tuberosus* populations in forest ecosystems of *A. araucana* and clarifies the relationships between these characteristics and the geographic distributions of the populations; accordingly, these findings extend our knowledge of *C. tuberosus* populations in Chile.

Conflicts of interest

The authors declare no conflicts of interest

Acknowledgments

This research was funded by FONDEF D10I1038 (Biodiversity information network to guide the properties of scientific research in support of public environmental policies) and DIUFRO DI14-0111 [Chemical and biological characterization of Maiten (*Maytenus boaria*) as a food attractant of *A. superciliosus* (Coleoptera: Curculionidae)]. DIUFRO DI13-TD01 supported the doctoral thesis.

References

Arias, E., 2000. Coleópteros de Chile. (Chilean Beetles), Primera Ed. Fototeknika, Santiago.
Barriga, J., et al., 1993. Nuevos antecedentes de coleópteros xilófagos y plantas hospederas en Chile, con una recopilación de citas previas. Rev. Chil. Entomol. 20, 65–91.
Bekessy, S.A., et al., 2002. Genetic variation in the vulnerable and endemic Monkey Puzzle tree, detected using RAPDs. Heredity 88, 243–249.
Bornet, B., Branchard, M., 2004. Use of ISSR fingerprints to detect microsatélites and genetic diversity in several related *Brassica* taxa and *Arabidopsis thaliana*. Hereditas 140, 245–247.
Donoso, C., et al., 2006. Variación Intraespecífica en Las Especies Arbóreas de Los Bosques Templados de Chile y Argentina, Primera Ed. Editorial Universitaria, Santiago.
Donoso, C., et al., 2008. Poblaciones de araucaria enana (*Araucaria araucana*) en la Cordillera de Nahuelbuta, Chile. Bosque 29, 170–175.
Elgueta, M., Marvaldi, A.E., 2006. Lista sistemática de las especies de Curculionoidea (Insecta: Coleoptera) presentes en Chile, con su sinonimia. Bol. Mus. Hist. Nat. Chile 55, 113–153.
Elgueta, M., 2008. Orden Coleóptera. In: Biodiversidad de Chile, Patrimonio y Desafíos, Segunda edición. Ocho Libros Editores, Santiago.
Elgueta, M., et al., 2008. Curculionoidea (Coleóptera) en follaje de árboles del centro-sur de Chile. In: Contribuciones Taxonómicas en Ordenes de Insectos Hiperdiversos. Llorente & Lanteri editores.
Fekrat, L., et al., 2014. Morphometric and molecular variation in *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) populations on onion and tobaco in Iran. J. Agric. Sci. Technol. 16, 1505–1516.
Hammer, O., et al., 2001. Paleontological statistics software package for education and data analysis. Palaeontol Electronica 4, 1–9.
Hechenleitner, P., et al., 2005. Plantas Amenazadas del Centro-sur de Chile. Distribución, Conservación y Propagación, Primera Ed. Universidad Austral de Chile (Valdivia) & Real Jardín Botánico de Edimburgo.
Herrmann, T.M., 2006. Indigenous knowledge and management of *Araucaria araucana* forest in Chilean Andes: implications for native forest conservation. Biodivers. Conserv. 15, 647–662.
Heusser, C.J., et al., 1988. Late-Holocene vegetation of the Andean *Araucaria* region province of Neuquén, Argentina. Mt. Res. Dev. 8, 53–63.
Joshi, S.P., et al., 2000. Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor. Appl. Genet. 100, 1311–1320.
Korpelainen, H., et al., 2007. Microsatellite marker identification using genome screening and restriction-ligation. Biotechniques 42, 479–486.
Kuschel, G., 2000. La fauna curculiónica (Coleoptera: Curculionoidea) de la *Araucaria araucana*. Rev. Chil. Entomol. 27, 41–51.
Lee, Y.H., Lin, C.P., 2012. Morphometric and genetic differentiation of two sibling gossamer-wing damselflies. *Euphaea formosa* and *E. yayeyamana*, and adaptive trait divergence in subtropical East Asian islands. J. Insect. Sci. 12, 1–17.
Marchelli, P., et al., 2010. Biogeographic history of the threatened species *Araucaria araucana* (Molina) K. Koch and implications for conservation: a case study with organelle DNA markers. Conserv. Genet. 11, 951–963.
Marconi, B.G., et al., 2011. Primer Note: microsatellite-AFLP development for *Araucaria araucana* (Mol.) K. Koch, an endangered conifer of Chilean and Argentinean native forests. Silvae Genet. 60, 285–288.
Martín, M.A., et al., 2014. New insights into the genetic structure of *Araucaria araucana* forest based on molecular and historic evidence. Tree Genet. Genomes, http://dx.doi.org/10.1007/s11295-014-0725-1.
Marvaldi, A.E., Lanteri, A.A., 2005. Key to higher taxa of South American weevils based on adult characters (Coleoptera Curculionoidea). Rev. Chil. Hist. Nat. 78, 65–87.
Moreno, A.C., et al., 2011. Cross transferability to SSRs to five species of Araucariaceae: a useful tool for population genetic studies in *Araucana araucana*. Forest Syst. 20, 303–314.
Morrone, J.J., 1997. Weevils (Coleóptera: Curculionoidea) that feed on *Araucaria araucana* (Araucariaceae) in southern Chile and Argentina, with an annotated checklist. Folia Entomol. Mex. 100, 1–14.
Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283–292.
Oberprieler, R.G., et al., 2007. Weevils, weevils, weevils everywhere. Zootaxa 1668, 491–520.
Olivero, P.A., et al., 2012. Morphometry and geographical variation of *Bothriurus bonariensis* (Scorpiones: Bothriuridae). J. Arachnol. 40, 113–122.
Olsen, G.J., Woese, C.R., 1993. Ribosomal RNA: a key to phylogeny. Faseb J. 7, 113–123.
Pérez de la Torre, M.C., 2012. Analysis of genetic variability by ISSR markers in *Calibrachoa caesia*. Electron. J. Biotechnol. 15, 1–12.
Posadas, P.E., et al., 2007. Dimorfismo sexual y variación morfométrica geográfica en *Hybreoleptops aureosignatus* (Insecta: Coleoptera: Curculionidae). An. Acad. Nac. de Cs. Ex. Fís. Nat. 59, 141–150.
Przybycien, M., Waclawik, B., 2015. Morphometric measurements of *Bryodaemon* (Coleoptera: Curculionidae): contribution to phylogeny. Baltic J. Coleopterol. 15, 129–136.
Rafii, Z.A., Dodd, R.S., 1997. Genetic diversity among coastal and Andean natural populations of *Araucaria araucana* (Molina) K. Koch. Biochem. Syst. Ecol. 6, 441–451.
Sánchez-Ruiz, M., San Martín, I., 2000. Separation of *Aspidiotus* species using morphometric analysis (Coleoptera: Curculionidae). Eur. J. Entomol. 97, 85–94.
Schwarzfeld, M.D., Sperling, F.A., 2014. Species delimitation using morphology, morphometrics, and molecules: definition of the *Ophion scutellaris* Thomson species group, with descriptions of six new species (Hymenoptera, Ichneumonidae). ZooKeys 462, 59–114.
Sequeira, A.S., Farrell, B.D., 2001. Evolutionary origins of Gondwanan interactions: how old are *Araucaria* beetle herbivores? Biol. J. Linn. Soc. 74, 459–474.
Sneath, P.H., Sokal, R.R., 1975. Numerical taxonomy. The principles and practice of numerical classification. Syst. Zool. 24, 263–268.
Soto, M., Reyes, P., 2014. Nuevo registro de dos especies de *Anthonomocyllus* (Curculionidae Anthonomini) para México. Rev. Colomb. Entomol. 40, 292–295.
Tikunov, Y.M., et al., 2003. Application of ISSR markers in the genus *Lycopersicon*. Euphytica 131, 71–80.
Vergara, O.E., et al., 2006. Diversidad y patrones de distribución de coleópteros en la región del Biobío. Chile: una aproximación preliminar para la conservación de la diversidad. Rev. Chil. Hist. Nat. 79, 369–388.
Villagrán, C., 2001. Un modelo de la historia de la vegetación de la Cordillera de La Costa de Chile central-sur: la hipótesis glacial de Darwin. Rev. Chil. Hist. Nat. 74, 793–803.
Wanek, K.A., Sturmbauer, C.H., 2015. Form, function and phylogeny: comparative morphometrics of Lake Tanganyika's Cichlid tribe Tropheini. Zool. Scr., http://dx.doi.org/10.1111/zsc.12110.
Woese, C.R., Fox, G.E., 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. U. S. A. 74, 5088–5090.
Wolodarsky-Franke, A., Díaz, S.A., 2011. Cordillera de Nahuelbuta. Reserva Mundial de Biodiversidad, Primera Ed. World Wildife Fund, Valdivia, Chile.
Wortley, A.H., Scotland, R.W., 2006. The effect of combining molecular and morphological data in published phylogenetic analyses. Syst. Biol. 55, 677–685.
Yang, Z., Rannala, B., 2012. Molecular phylogenetics: principles and practice. Nat. Rev. 13, 303–314.
Yua, T., 2011. Genetic variation and clonal diversity of *Bromus ircutensis* Kom in the Otingdag Sandy Land detected by ISSR markers. Genetika 47, 796–804.
Yeh, F.C., et al., 1999. POPGENE, Version 1.31. University of Alberta, Edmonton, Canada, Available from https://sites.ualberta.ca/~fyeh/popgene.html (accessed 13.08.17).
Zietkiewicz, E., et al., 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20, 176–183.