Within-population genetic diversity of climbing plants and trees in a temperate forest in central Chile

Diversidad genética intra-poblacional de plantas trepadoras y árboles en un bosque templado en Chile central

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ABSTRACT

The climbing habit is a key innovation in angiosperm evolution: climbing plant taxa have greater species richness than their non-climbing sister groups. It is considered that highly diversified clades should show increased among-population genetic differentiation. Less clear is the expected pattern regarding within-population genetic diversity in speciose lineages. We tested the hypothesis of greater within-population genetic diversity in climbing plants compared to trees in a temperate forest in central Chile. The assumption underlying this hypothesis is that higher among-population differentiation in climbers compared to trees should reflect higher genetic diversity as well. AFLP markers from 167 individual plants from 14 species (seven climbers and seven trees) were used to estimate the following indices of within-population genetic diversity: mean unbiased expected heterozygosity (H_E), percentage of polymorphic loci (PPL), Shannon information index (I), and the effective number of alleles (N_E). Overall, within-population genetic diversity did not differ between climbing plants and trees. The H_E for climbing plants was slightly higher than that of trees (0.247 vs. 0.231), and PPL was higher in trees (93.6) than in climbers (81.8), but these differences were not statistically significant. Both I and N_E were very similar for both groups. The expected greater genetic diversity in climbers might have been counterbalanced by tree-related ecological factors that turned to be relevant in the species assemblages studied. Results of this preliminary study should be further confirmed with a broader approach including several forest sites and larger sample sizes.

Keywords: AFLP, climbers, evolution, genetic variability, key innovation.

RESUMEN

El hábito trepador es una innovación clave en la evolución de las angiospermas: los taxa de plantas trepadoras poseen mayor riqueza de especies que sus grupos hermanos no-trepadores. Se considera que clados altamente diversificados debieran mostrar mayor diferenciación genética entre poblaciones. Menos claro es el patrón esperado respecto a la diversidad genética al interior de las poblaciones en linajes especiosos. Evaluamos la hipótesis de mayor diversidad genética intra-poblacional en plantas trepadoras en comparación con árboles en un bosque templado en Chile central. El supuesto detrás de esta hipótesis es que la mayor diferenciación poblacional en las trepadoras, comparadas con los árboles, debiera reflejar una mayor diversidad genética también. Marcadores AFLP de 167 plantas individuales de 14 especies (siete trepadoras y siete árboles) fueron usados para estimar los siguientes índices de diversidad genética intra-poblacional: heterocigosidad esperada media (H_E), porcentaje de loci polimórficos (PPL), índice de información de Shannon (I), y el número efectivo de alelos (N_E). En general, la diversidad genética intra-poblacional no difirió entre plantas trepadoras y árboles. La H_E para plantas trepadoras fue levemente mayor a la de los árboles (0,247 vs. 0,231), y el PPL fue más alto en árboles (93,6) que en trepadoras (81,8), pero estas diferencias no fueron estadísticamente significativas. Tanto I como N_E fueron muy similares en ambos grupos. La mayor diversidad genética esperada en trepadoras puede hacer sido contrarrestada por factores ecológicos particulares a los árboles que resultaron ser relevantes en los ensambles de especies estudiados. Los

resultados de este estudio preliminar debieran ser confirmados con una aproximación a mayor escala, incluyendo varios sitios en el bosque y mayores tamaños muestrales.

Palabras clave: AFLP, innovación clave, evolución, trepadoras, variabilidad genética.

INTRODUCTION

Angiosperms, with c. 300,000 species, appear to be the most successful and dominant group of land plants and have undergone an outstanding diversification compared to other plant groups (Stebbins 1981, Crane *et al*. 1995, Magallón & Castillo 2009). The evolutionary success of certain lineages within angiosperms has been related to a number of plant features, including life history traits, growth habits, specialized organs, and physiological pathways (Quezada & Gianoli 2011 and references cited therein). Although taxa diversification cannot be evaluated in ecological time scales, it is considered that genetic differentiation among populations may be a surrogate for speciation (Grant 1981, Avise 2000, Levin 2000). For instance, the hypothesis that increased speciation rate explains the greater species richness in the tropics has been tested and verified using population differentiation within plant species (Eo *et al*. 2008) and vertebrate species (Martin & McKay 2004) as a proxy for speciation. Moreover, Funk *et al*. (2006) have shown that ecological divergence is consistently and positively associated with reproductive isolation across a number of taxa, including plants and animals.

The evolution of a climbing habit has taken place independently in more than 130 plant families (Gentry 1991) and it seems to be a key innovation (Hunter 1998) in angiosperms: climbing plant taxa have greater species richness than their non-climbing sister groups (Gianoli 2004). A similar result was found for epiphytism: epiphytic genera were significantly richer in species than terrestrial genera, both for orchids and non-orchids (Gravendeel *et al*. 2004). Which features of the climbing habit, measurable at an ecological timescale, may eventually promote plant diversification? In contrast with either erect or prostrate species, which occupy a relatively narrow range of the light gradient, climbers may use a very broad range of light availability through both supported (climbing) and unsupported (creeping) individuals. With the occupation of such an expanded ecological niche –ranging from forest floor to understory to forest canopy– a greater exposure to different pollinators, fruit/seed dispersers, and herbivores would be granted and hence ecological specialization might be favoured (Gianoli 2004). This is somewhat analogous to the prevailing explanation for the outstanding diversification of Angiosperms (Crepet & Niklas 2009).

Genetic differentiation among populations within species is considered as a first step towards speciation, and therefore highly diversified clades should show increased between-population genetic differentiation (Grant 1981, Avise 2000, Levin 2000, Coyne & Orr 2004). Less clear is the expected pattern regarding within-population genetic diversity in speciose lineages. Increased genetic differentiation among populations is often associated with reduced genetic diversity within populations because of contrasting effects of several factors, including breeding system, longevity, floral morphology, gene flow, mode of reproduction, pollination and seed dispersal mechanisms, and fragmentation (Loveless & Hamrick 1984, Hamrick & Godt 1996, Austerlitz *et al*. 2000, Eckstein *et al*. 2006). In the case of the effects of habitat fragmentation, initial assumptions that fragmentation should lead to both reduced genetic diversity and increased population divergence due to increased genetic drift, high inbreeding and reduced gene flow, have been relaxed in view of empirical evidence (Young *et al*. 1996, Wagner *et al*. 2011). In fact, considerable genetic differentiation among populations may coexist with high levels of within-population genetic diversity (Ægisdóttir *et al*. 2009). Alternatively, high genetic diversity within populations may also be hypothesized to facilitate among-population genetic differentiation, which may putatively explain the higher diversification rates of some lineages. This assumes that the process of divergent selection, which may lead to genetic differentiation between populations, is fostered when genetic diversity within the population is significant (e.g. Kärkkäinen *et al*. 2004). However, it should not be overlooked that after natural selection takes place genetic diversity within populations tends to be reduced (Gillespie 2000). Interestingly, Segarra-Moragues & Ojeda (2010) studied how plant responses to fire may explain the high levels of diversification in *Erica*. They found that seeder populations (fire sensitive) harbour higher levels of among-population and within-population genetic variation than resprouter populations (fire resistant). This was attributed to the shorter generation time and thus faster population turnover in seeders, which would lead to higher mutation rates and faster generation of new alleles (Segarra-Moragues & Ojeda 2010).

The aim of the present study was to test the hypothesis of greater within-population genetic diversity in climbing plants compared to trees. This follows the finding that the climbing habit is a key innovation that promotes diversification in angiosperm clades (Gianoli 2004). In a forest fragment located in the temperate-Mediterranean transition zone in central Chile, we quantified within-population genetic diversity indices of seven climbing plant species and seven tree species using AFLP markers.

MATERIALS AND METHODS

Study site and species

The study was carried out in the private reserve Parque Coyanmahuida (36°49'S, 72°43'W; 250-300 m a.s.l), which is a 24-ha fragment of native forest with both Mediterranean and temperate floristic components, surrounded by pine plantations. The study site is located 27 km east of the city of Concepción, which has a mean annual precipitation of ~1200 mm with a marked decrease in rainfall during the austral summer months (Parada & Lusk 2011).

The 14 study species are the most common trees and climbers in the forest fragment and are listed in Table 1, together with some biological features. Taxonomic treatments follow Marticorena & Quezada (1985) and the Angiosperm Phylogeny Group (Stevens 2011).

Sampling

Plant material was collected after random walks along paths and across the forest interior. To avoid sampling a single genotype more than once because of vegetative reproduction, all sampled conspecific individuals were at least 10 m apart. Sample size ranged from five to 16 individuals per species. Approximately 100 mg (fresh weight) of young leaves were collected from each individual plant. Samples were dried in bags with silica gel and stored at 4 °C until DNA extraction.

Total DNA extraction and AFLP protocol

Total DNA was extracted from dry leaf material according to the protocol of Doyle & Doyle (1987) and treated with RNAse (30 min at 37 ºC). AFLP profiles were obtained following the original procedures of Vos *et al*. (1995) and according to the modifications described in Hasbún *et al*. (2012). First, the digestion and ligation of genomic DNA were performed in a single step. Each reaction was conducted in a 11 µl volume containing 10-50 ng of DNA, 1x T4 DNA ligase buffer NEB (50 mM Tris HCl, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP), 55 ng BSA, 50 uM NaCl, 5 U *EcoRI* (NEB), 1 U *Mse I* (NEB), 10 cohesive units of T4 DNA ligase, 5 pmol EcoRI adaptor and 50 pmol MseI adaptor. That mixture was incubated for 2 h at 37 ºC and diluted to 22 µl in distilled water and stored at -20 ºC. Preselective amplification reaction was performed using 3 µl of the diluted digested-ligated DNA in a total volume of 25 µl. The mixture contained 1xPCR buffer, 1.5 mM $MgCl_2$, 0.12 mM of each dNTP, 0.2 µM Eco+A primer, 0.2 µM Mse+A (5′-GATGAGTCCTGAGTAA+A) primer and 0.5 U *Taq DNA polymerase* (Invitrogen, Sao Paulo, Brazil) (M+C and E+A). PCR amplification was carried out with an initial denaturation of 2 min at 94 ºC, and then 25 cycles of 20 s at 94 ºC, 20 s at 60 ºC and 2 min at 72 ºC, followed by a final step of 30 min at 60 ºC. After PCR amplification products were diluted 1:10 with distilled water and stored at -20 ºC. Selective amplification was performed using 2.5 µl of diluted preselective amplification as a template in reactions containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.1 µM Eco+ ANN (fluorescent) primer, 0.5 µM Mse+2 or Mse+3 primer, and 0.5 U Platinum Taq DNA polymerase in a final volume of 12.5 µl. Amplifications were performed with an initial denaturation of 2 min at 94 ºC, then 30 cycles of 20 s at 94 ºC, 20 s at 66 ºC and 2 min at 72 ºC, followed by a final step of 30 min at 60 ºC to avoid split peaks.

A series of selective combinations were tested for each species including M+CN, M+CNN and E+ANN. Selective oligonucleotides were synthesized and labelled at the 5'-end with a fluorescent dye at Applied Biosystems Custom Oligonucleotide Synthesis Service (Applied Biosystems Inc, Foster City, CA, USA). All selective profiles were run in a Genetic Analyzer Sequencer (Applied Biosystems) at the Laboratorio de Genómica Forestal, Centro de Biotecnología, Universidad de Concepción. Screening for selective primer combination was assayed to obtain the best banding patterns. To confirm reproducibility and reliability of AFLP markers, five duplicated reactions per species were run and those markers that showed inconsistent amplifications were not scored. The selective amplification products were run in an automatic sequencer (Applied Biosystem 3130, 16 capillaries) at the Laboratorio de Genómica Forestal, Centro de Biotecnología, Universidad de Concepción. The clearest and most intense polymorphic bands were coded in a presence/absence (1/0) matrix.

Statistical analysis

The average unbiased expected heterozygosity (H_E), Shannon information index (I), percentage of polymorphic loci (PPL), and the effective number of alleles (N_E) were calculated for each species assuming Hardy-Weinberg equilibrium using GENALEX version 6.1 (Peakall & Smouse 2006). To test whether genetic diversity is higher in climbing plant species than in tree species we used *t*-tests as all genetic diversity statistics showed a normal distribution and variance homogeneity. This analysis was performed using Statistica 6.0 (Statsoft, Tulsa, OK, USA). We did not form pairs of phylogenetically related climber and tree species because families and orders of climbers and trees were too phylogenetically distant (see Table I).

RESULTS

A single primer combination (mostly Eco+3/Mse+3) produced enough polymorphism to quantify within-population genetic diversity for all studied species. The number of scored bands ranged from 72 to 262 in climbing species, and from 107 to 296 in tree species (Table II). The final number of genotyped plants ranged from 5 to 15 for climbing plant species and between 9 and 16 individuals for tree species (Table III).

The mean unbiased expected heterozygosity (H_E) for climbing plants was slightly higher than that of trees (0.247 vs. 0.231), but this difference was not statistically significant (t = -0.75, P = 0.47; Table III). The percentage of polymorphic loci (PPL) was higher in trees (PPL = 93.6) than in climbers (PPL = 81.8), this difference being marginally non-significant (t = 2.09, P = 0.06; Table III). Both the Shannon information index (I) and the effective number of alleles (N_E) were very similar for climbing plants and trees (Table III) (t = -0.19, P = 0.85, and t = -0.38, P = 0.71, respectively).

Table I. Study species, their taxonomic categories and some biological features.

Tabla I. Especies de estudio, sus categorías taxonómicas y algunas características biológicas.

Species	Family	Order	Life form	Fruit	Flower
Climbers					
Bomarea salsilla (L.) Mirb.	Alstroemeriaceae	Liliales	herbaceous	dry	Hermaphroditic
Boquila trifoliolata (DC.) Decne.	Lardizabalaceae	Ranunculales	woody	fleshy	Monoecious
Cissus striata Ruiz et Pav.	Vitaceae	Vitales	woody	fleshy	Hermaphroditic
Dioscorea humifusa Poepp.	Dioscoreaceae	Dioscoreales	herbaceous	dry	Dioecious
Lapageria rosea Ruiz et Pav.	Philesiaceae	Liliales	woody	fleshy	Hermaphroditic
Lardizabala biternata Ruiz et Pav.	Lardizabalaceae	Ranunculales	woody	fleshy	Dioecious
Muehlenbeckia hastulata (Sm.) I.M. Johnst.	Polygonaceae	Caryophyllales	woody	dry	Monoecious
Trees					
Aextoxicon punctatum Ruiz et Pav.	Aextoxicaceae	Berberidopsidales	woody	fleshy	Dioecious
Aristotelia chilensis (Molina) Stuntz	Elaeocarpaceae	Oxalidales	woody	fleshy	Dioecious
Citronella mucronata (Ruiz et Pav.) D. Don.	Cardiopteridaceae	Aquifoliales	woody	fleshy	Hermaphroditic
Cryptocarya alba (Molina) Looser	Lauraceae	Laurales	woody	fleshy	Hermaphroditic
Luma apiculata (DC.) Burret	Myrtaceae	Myrtales	woody	fleshy	Hermaphroditic
Peumus boldus Molina	Monimiaceae	Laurales	woody	fleshy	Dioecious
Rhaphithamnus spinosus (Juss.) Moldenke	Verbenaceae	Lamiales	woody	fleshy	Hermaphroditic

Table II. Selective primer combinations, number of scored bands and number of polymorphic loci in the study species.

Tabla II. Combinaciones de partidores selectivos, número de bandas registradas y número de loci polimórficos en las especies de estudio.

Species	Selective primer combination	Total number of bands	Number of polymorphic bands
Climbers			
Bomarea salsilla	M+CAT / E+AGC	244	226
Boquila trifoliolata	M+CAT / E+AGA	72	53
Cissus striata	M+CA / E+AGC	221	217
Dioscorea humifusa	M+CAT / E+AGA	162	151
Lapageria rosea	M+CAT / E+AGA	262	207
Lardizabala biternata	M+CAT / E+AGA	76	48
Muehlenbeckia hastulata	M+CA / E+AGA	96	70
Trees			
Aextoxicon punctatum	M+CAT / E+AGA	115	89
Aristotelia chilensis	M+CAT / E+AGA	107	106
Citronella mucronata	M+CAT / E+AGA	200	186
Cryptocarya alba	M+CAT / E+AGA	157	156
Luma apiculata	M+CAT / E+AGA	218	208
Peumus boldus	M+CAT / E+AGA	296	291
Rhaphithamnus spinosus	M+CAT / E+AGA	117	110

TABLE III. Within-population genetic diversity statistics for the study species. No significant differences were found between groups (climbers vs. trees); see text for details.

TABLA III. Estadísticas de diversidad genética intra-poblacional para las especies de estudio. No se encontraron diferencias significativas entre los grupos (trepadoras vs. árboles); ver detalles en el texto.

CLIMBERS	*N*	H_E	PPL	*I*	N_E	TREES	*N*	H_E	PPL	*I*	N_E
Bomarea salsilla	15	0.255	92.6	0.385	1.832	*Aextoxicon punctatum*	15	0.227	77.4	0.339	1.774
Boquila trifoliolata	7	0.221	73.6	0.323	1.325	*Aristotelia chilensis*	9	0.286	98.1	0.419	1.441
Cissus striata	12	0.215	98.0	0.340	1.311	*Citronella mucronata*	15	0.202	93.0	0.320	1.195
Dioscorea humifusa	15	0.307	93.2	0.442	1.472	*Cryptocarya alba*	15	0.225	99.3	0.349	1.994
Lapageria rosea	15	0.212	79.0	0.320	1.286	*Luma apiculata*	12	0.211	95.4	0.336	1.314
Lardizabala biternata	6	0.212	63.1	0.299	1.325	*Peumus boldus*	16	0.201	98.3	0.323	1.298
Muehlenbeckia hastulata	5	0.305	72.9	0.405	1.484	*Rhaphithamnus spinosus*	10	0.267	94.0	0.396	1.407
Mean	10.7	0.247 (0.02)	81.8 (4.91)	0.359 (0.02)	1.424 (0.07)		13.1	0.231 (0.05)	93.6 (1.07)	0.355 (0.01)	1.489 (0.04)

N = sample size, H_E = unbiased expected heterozygosity, PPL = percentage of polymorphic loci, *I* = Shannon's information index, N_E = No. of effective alleles. Values in the last row are means (SE)

DISCUSSION

The results show that, overall, the levels of within-population genetic diversity do not differ between climbing plants and trees in the studied forest fragment. Thus, we did not find support for the hypothesis that there would be a greater potential for within-population genetic variation in climbing plants than in trees, which was based on the reported greater species richness of climbing clades compared to their non-climbing sister groups (Gianoli 2004). The levels of within-population genetic diversity found in climbing plants (0.247) and trees (0.231), measured as expected heterozygosity (H_E), were similar to those found in long-lived perennials (Hamrick & Godt 1996, Nybom 2004).

Several possible explanations for the observed lack of differences between climbers and trees may be analyzed. It might be hypothesized that the expected greater genetic diversity in climbers, underlying the macroevolutionary pattern reported earlier (Gianoli 2004), was counterbalanced by ecological factors that turned to be relevant in the species assemblages studied. First, there is evidence of higher genetic diversity in long-lived perennials than in short-lived perennials (Nybom 2004) and, likewise, that woody species tend to show more genetic diversity within populations than herbaceous species (Hamrick & Godt 1996, Austerlitz *et al*. 2000). We lack information about species longevity in the study site, but given that the climbing plant group included two herbaceous species, presumably short-lived, such an explanation for a relative "advantage" of trees could be initially considered. However, those two species (*Bomarea* and *Dioscorea*) actually had genetic diversity levels above the average for the group. Therefore, this explanation is unlikely. Second, whereas climbers had three species with dry fruits, all tree species had fleshy fruits. This could be considered to have an impact on within-population genetic diversity, via differential seed dispersal distance. However, it has been shown that there is no clear-cut pattern of association between seed dispersal mechanisms and genetic diversity, the advantages of fleshy fruits being rather dependent on the expression of other life history traits (Hamrick & Godt 1996). Interestingly, it has been reported at the macroevolutionary level that clades with fleshy fruits are significantly more diverse than sister clades with dry fruits (Smith 2001). Third, it has been documented that higher levels of genetic variation within populations are expected in dioecious or monoecious species compared to those with hermaphroditic flowers (Loveless & Hamrick 1984). However, in the present case no obvious difference in the relative proportion of species with either floral type was found between climbing plants and trees. Therefore, this factor cannot account for the supposed counterbalance to the intrinsically high diversification potential of climbers that would be expressed at the within-population scale.

The results of the study may also be explained by the small sample size (seven species per group) and hence reduced statistical power to detect differences. Nonetheless, the sample of plant species is representative of the study site, which is not particularly species-rich (Parada & Lusk 2011). The relatively low number of replicates within species may have also influenced the results, but there were not too many plants available (and DNA could not be extracted in several cases). Anyway, results were obtained from a single forest fragment and a broader sampling is needed to draw general conclusions. Further investigations will include as study sites several populations within the continuous temperate rainforest, with greater species richness of both climbing plants and trees (Gianoli *et al*. 2010, Valladares *et al*. 2012), which should allow a better evaluation of the research hypothesis. Moreover, we plan to address a complementary question along the general research line, i.e., the testing of the key innovation hypothesis regarding the climbing habit at an ecological time scale. Thus, further research will evaluate whether climbing plants have undergone greater population differentiation than trees in the southern temperate rainforest (Valdivian forest). In this regard, the endemic climbing plant *Berberidopsis corallina* (Berberidopsidaceae) showed a markedly high level of genetic differentiation between populations (Etisham-Ul-Haq *et al*. 2001), far greater than those reported for native Chilean trees such as *Pilgerodendron uviferum* (Cupressaceae) (Allnutt *et al*. 2003), *Fitzroya cupressoides* (Cupressaceae) (Allnutt *et al*. 1999), *Podocarpus salignus* (Podocarpaceae) (Allnutt *et al*. 2001), *Gomortega keule* (Gomortegaceae) (Herrera *et al*. 2005) and *Aextoxicon punctatum* (Aextoxicaceae) (Núñez-Avila & Armesto 2006) comparing populations more geographically distant than those included in the *B. corallina* study. These antecedents strongly suggest that the general question put forward deserves further attention.

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