

PHYLOGENETIC RELATIONSHIPS AMONG CHILEAN SPECIES OF *DRIMYS*  
(WINTERACEAE) BASED ON ITS SEQUENCES AND INSERTION/DELETION  
EVENTS

*RELACIONES FILOGENETICAS ENTRE LAS ESPECIES CHILENAS DE DRIMYS*  
(WINTERACEAE), BASADAS EN SUS SECUENCIAS ITS Y EVENTOS DE  
INSERCIÓN/DELECIÓN

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ABSTRACT

Phylogenetic relationships among Chilean species of *Drimys* as well as *D. granadensis* from Central America and *D. angustifolia* from Brazil were reconstructed using internal transcribed spacers (ITS) of nuclear ribosomal DNA and a parsimony analysis. Our results at the family level were congruent with previous phylogenetic hypotheses. Molecular data indicate that the Juan Fernandez Island endemic *D. confertifolia* diverged early in the evolution of the genus, and it is not closely related to the continental species *D. winteri*, as might be expected on the basis of morphology and biogeography. The ITS phylogeny also provides general support for a recent distribution of species on the western side of the Andes Mountains; the only exception is *D. andina*, which may represent an early divergence that occurred just before the Quaternary. The only species examined from east of the Andes, *D. angustifolia*, also represents an early divergence in the genus, which is in agreement with the ancient pollen record in Argentinean Patagonia and the lack of Tertiary records in Chile.

KEYWORDS: *Drimys*, evolution, ITS, indels, Winteraceae.

RESUMEN

Se reconstruyeron relaciones filogenéticas de las especies chilenas de *Drimys*, que incluyó a *D. granadensis* de América Central y *D. angustifolia* de Brasil, donde se usó la región del espaciador transcrito interno (ITS) del ADN ribosomal nuclear y sus eventos de inserción/delección (indels) a través de un análisis de parsimonia. Nuestros resultados a nivel de familia fueron congruentes con previas hipótesis filogenéticas propuestas. Los datos moleculares indican que *D. confertifolia*, endémica del Archipiélago de Juan Fernández, divergió temprano en la evolución del género, y que no está cercanamente emparentada a las especies continentales de *D. winteri*, como se podría esperar sobre la base de la morfología y la biogeografía. Los datos moleculares también apoyan una distribución reciente en el lado oeste de la Cordillera de los Andes, la única excepción es *D. andina* la cual puede representar una divergencia temprana que ocurrió justo antes del Cuaternario. La única especie estudiada del lado este de la Cordillera de los Andes, *D. angustifolia*, también representa una divergencia temprana en el género, hipótesis que concuerda con el antiguo registro palinológico del Terciario en la Patagonia argentina y la falta de estos en Chile.

PALABRAS CLAVE: *Drimys*, evolución, ITS, inserciones, Winteraceae.

## INTRODUCTION

*Drimys* J.R.Forst. et G.Forst. (Winteraceae) is distributed exclusively in Central and South America including the Juan Fernández Island and comprises 5 to 7 species (Smith 1943, Ehrendorfer *et al.* 1979, Vink 1993, Rodríguez & Quezada 2001). The genus formerly included the species from Australasia as a Sect. *Tasmannia* (DC.) F.Muell (Vink 1970, 1993). However, recent studies have led to split the genus into two, with New World species remaining in genus *Drimys*, and the Australasian species classified in genus *Tasmannia* (Doust & Drinnan 2004). The relationships among species have been unclear, which has resulted in different perspectives on the limits of species and varieties. At one extreme, Hooker (1845) regarded all central and South American taxa as belonging to a single species. At the other extreme, Miers (1858, 1862) recognized nine species in the New World. An intermediate view was proposed by Smith (1943), who recognized four species, three of which possessed infraspecific taxa (i.e., vars.). Ehrendorfer *et al.* (1979) based on morphological traits, recognized *D. angustifolia* Miers and described a new combination, *D. roraimensis* (A.C.Smith) Ehrend. et Gliottsb. from Brazil. The taxonomic treatment accorded the Chilean (and adjacent Argentinean) members of *Drimys* has varied depending on the worker. Currently three species are recognized in Chile (Rodríguez & Quezada 2001): *Drimys andina* (Reiche) R.A.Rodr. et Quez., a shrub restricted to higher parts of the Coastal and Andes mountains (37°43' -41°34'S), and two arborescent species, *Drimys confertifolia* Phil. endemic to the Juan Fernandez Islands and *Drimys winteri* J.R.Forst. et G.Forst. with two varieties, *D. winteri* var. *winteri* occurring in Southwestern Patagonia (45°44' -55°58'S) and *D. winteri* var. *chilensis* (DC.) A.Gray being widespread in Chile and Argentina (30°20' -46°25'S) (Fig. 1). The island endemic has been treated as a distinct species (Philippi 1856, Miers 1858, Smith 1943, Rodríguez & Quezada 2001) or as a variety of *D. winteri* (Reiche 1895, Johow 1896, Skottsberg 1922). Likewise, *D. andina* has been considered as a variety of *D. winteri* (Philippi 1856, Urban 1934, Smith 1943, Muñoz 1980, Marticorena & Quezada 1985). Both Ehrendorfer *et al.* (1979) and Smith (1943) considered both taxa (*D. andina* and *D. winteri*) to have differentiated due to the uplift of the Andean Mountains. Recently, principally based on morphology, geographical distribution, and ecology,

*D. andina* has been considered a distinct species (Rodríguez & Quezada 1991, 2001).

Phenetic analyses of the distribution of flavonoid compounds have shown that *D. andina* has the most distinct array of flavonoid constituents. The flavonoid patterns are congruent with current recognition of *D. andina* and *D. confertifolia* as distinct species (Ruiz *et al.* 2002).

Several recent phylogenetic studies of Winteraceae have been carried out using DNA sequences. However, these studies are of limited utility for examining patterns of evolution in *Drimys*, because only one (Suh *et al.* 1993, Karol *et al.* 2000) or two (Doust & Drinnan 2004) species of *Drimys* were

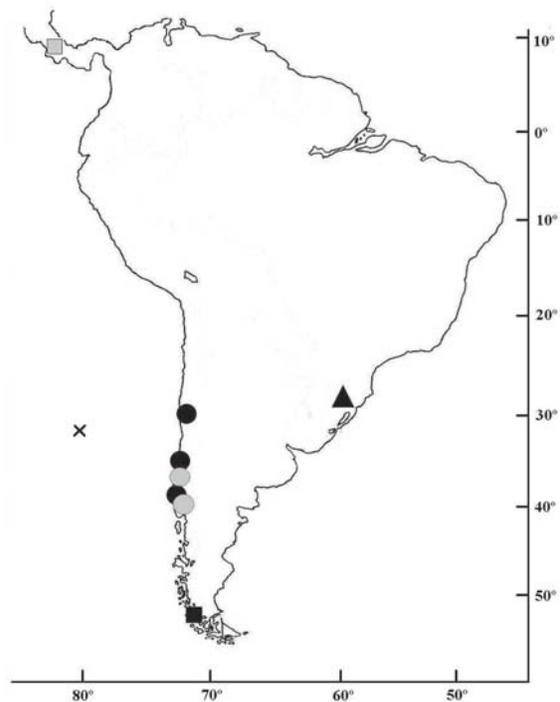


FIGURE 1. Distribution map of species of *Drimys* used in the current study. The black circles represent *D. winteri* var. *chilensis*, shaded circles: *D. andina*, black square: *D. winteri* var. *winteri*, shaded square: *D. granadensis*, black triangle: *D. angustifolia* and cross: *D. confertifolia*.

FIGURA 1. Mapa de distribución de las especies de *Drimys* usadas en este trabajo. Los círculos negros representan a *D. winteri* var. *chilensis*, círculos sombreados: *D. andina*, cuadrado negro: *D. winteri* var. *winteri*, cuadrado sombreado: *D. granadensis*, triángulo negro: *D. angustifolia*, y cruz: *D. confertifolia*.

included. Therefore, the purpose of the present study was to produce a hypothesis of phylogenetic relationships in *Drimys*, and to use the phylogeny as a framework to discuss the biogeography of the genus.

## MATERIAL AND METHODS

### PLANT MATERIAL

Material of *D. andina*, *D. confertifolia*, *D. winteri* var. *chilensis*, *D. winteri* var. *winteri*, was obtained from the field. Material of *Drimys angustifolia* from Brazil, was kindly sent to us by Dr. Leslie Landum from Arizona State University (ASU). The collected samples were deposited in the herbarium of University of Concepcion (CONC).

### PLANT MATERIAL STUDIED.

*Drimys andina*: CHILE. 1. Región de los Lagos, Osorno, upon Volcán Casablanca the limit of the vegetation, Nov. 1997, Ruiz 361 (CONC). 2. Región de la Araucanía, Prov. Malleco, Parque Nacional Nahuelbuta, Piedra del Aguila, 1350 m, Nov. 1985, Rodríguez *et al.* 2038 (CONC).

*Drimys confertifolia* CHILE. 1. Región de Valparaíso, Juan Fernández, Más Afuera, up to Cordón Atravesado, in Canelo Bajo, Nov. 1998, Ruiz 783 (CONC). 2. Juan Fernández, Más a Tierra, Salsipuedes ridge 470 m, Jan. 1997, Stuessy *et al.* 15239 (CONC).

*Drimys winteri* var. *chilensis* CHILE. 1. Región de Coquimbo, 5 km North from Los Vilos, small wet forest in the coastal side of the highway, Sept. 1997, Ruiz 301 (CONC). 2. Región de Bío-Bío, between Concepción and Santa Juana, Patagual bridge, trail to Coronel, two km toward Coronel, Oct 1997, Ruiz 330 (CONC). 3. Región de la Araucanía, Pto. Saavedra, from Pelarco, 2 km to the South, Nov 1997, Ruiz 389 (CONC).

*Drimys winteri* var. *winteri*. CHILE. Región de Magallanes. Provincia de Magallanes. Punta Arenas, Sector Faro San Francisco, en bosque costero de *Nothofagus*, 4 m, Feb 2006, Baeza 40a (CONC).

*Drimys angustifolia*. BRAZIL. RS- Mum. Bom Jesus-Fazenda Carúno, 1200 m, Jan 1997, R. Wasum & R.C. Molon s/n (ASU).

### DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total DNA was extracted from young leaf tissue using the CTAB method of Doyle & Doyle (1987). Double-stranded DNA of the complete region (ITS-1, 5.8S and ITS-2) was amplified by a symmetric PCR using White's universal ITS primers (1990): ITS 2 and 5, and ITS 3 with ITS 4 when necessary. The PCR products were obtained using the following reaction conditions: The 25 $\mu$ L PCR reaction contained 17.125 $\mu$ L sterile water, 2.5 $\mu$ L 10X Taq polymerase reaction buffer, 50mM magnesium chloride, 1 $\mu$ l of each primer (0.4  $\mu$ M), 0.125 units Taq polymerase and 2-10 ng template total DNA. In addition, 1 $\mu$ L DMSO was added to both the labelling and termination reactions to reduce the effects of secondary structure (Cosner *et al.* 1994). The first cycle consisted of 4 min 30 sec at 95 °C to denature template DNA, 1 min at 45 °C to anneal primers and 1 min at 72 °C to extend primers. Denaturing time was reduced to 1 min and the annealing temperature increased to 48 °C for each of the next 34 cycles. A final extension period of 9 min at 72 °C, terminated the PCR reaction. The amplification products were purified using QIAquick PCR Purification Kit (Quiagen) following the protocols suggested by manufacturers.

All the samples, except for *D. angustifolia* and *D. winteri* var. *winteri*, were directly sequenced by a cycle-sequencing ABI 377 automated sequencer (Applied Biosystem, Inc.), following the manufacturer's protocols. The *D. angustifolia* and *D. winteri* var. *winteri* ITS samples were sequenced in CESAT (Centro de Equipamiento y Servicio Tecnológico) at the University of Chile. All *Drimys* sequences were aligned with Clustal X (Thompson *et al.* 1997) with manual adjustment of the alignment by eye, and compared with the general matrix of Winteraceae and Canellaceae published by Karol *et al.* (2000) available in the web on TreeBASE ([www.treebase.org/treebase](http://www.treebase.org/treebase)). The current *Drimys* sequences are also available in Genbank (Table I).

### DATA MATRIX

The ITS sequences of *D. granadensis*, *Tasmannia xerophila* (P.Parm) Gray, *Tasmannia stipitata* (Vickery) A.C.Smith, *Tasmannia glaucifolia* J.B.Williams and *Tasmannia purpurescens* (Vickery) A.C.Smith were obtained from GenBank (Table I). The indel data were coded as presence/absence (0 = absent, 1 = present) following Simmons & Ocheterena (2000) and added to the matrix for maximum parsimony (MP) analysis. These results were subsequently

TABLE I. List of taxa used and Genbank accession numbers.

TABLA I. Lista de taxa utilizados y sus números de acceso a Genbank.

Species	Citation / Genbank accession number ITS
WINTERACEAE	
<i>Drimys andina</i> (Reiche) R.A.Rodr. et Quez.	This paper/ ITS1= EU683899 - ITS2=EU683900
<i>Drimys angustifolia</i> Miers	This paper/ITS1= EU683903 – ITS2= EU683904
<i>Drimys confertifolia</i> Phil.	This paper/ITS1= EU683901 - ITS2= EU683902
<i>Drimys granadensis</i> L.f.	Doust & Drinnan, 2004 / AY526318
<i>Drimys winteri</i> J.R.Forst. et. G.Forst. var. <i>chilensis</i> (DC.) A.Gray	This paper/ ITS1= EU683905 – ITS2= EU683906
<i>Drimys winteri</i> J.R.Forst. et G.Forst. var. <i>chilensis</i> (DC.) A.Gray (2 clones)	Suh <i>et al.</i> 1993
<i>Drimys winteri</i> J.R.Forst. et G.Forst. var. <i>winteri</i>	This paper/ ITS1= EU683907 – ITS2= EU683908
<i>Tasmannia glaucifolia</i> J.B.Williams	Doust & Drinnan, 2004 / AY526215
<i>Tasmannia insipida</i> R.Br. ex DC.	Suh <i>et al.</i> 1993 / AY004117
<i>Tasmannia lanceolata</i> (Poir.) A.C.Smith	Suh <i>et al.</i> 1993 / AY004145
<i>Tasmannia purpurescens</i> (Vickery) A.C.Smith	Doust & Drinnan, 2004 / AY526314
<i>Tasmannia stipitata</i> (Vickery) A.C.Smith	Doust & Drinnan, 2004 / AY526316
<i>Tasmannia xerophila</i> (P. Parm.) Gray	Doust & Drinnan, 2004 / AY526317
<i>Takhtajania perrieri</i> (Capuron) Baranova et J.-F.Leroy	Karol <i>et al.</i> 2000 / AY004129
<i>Pseudowintera axilaris</i> (J.R.Forst. & G.Forst.) Dandy	Suh <i>et al.</i> 1993 / AY004124
<i>Pseudowintera colorata</i> (Raoul) Dandy	Suh <i>et al.</i> 1993 / AY004125
<i>Bubbia comptoni</i> (Baker f.) Dandy	Suh <i>et al.</i> 1993 / AY004123
<i>Exospermum stipitatum</i> (Baill.) Tiegh. ex Morot.	Suh <i>et al.</i> 1993 / AY004121
<i>Zygogynum acsmithii</i> Vink	Suh <i>et al.</i> 1993 / AY004122
<i>Zygogynum balansae</i> Tiegh.	Suh <i>et al.</i> 1993 / AY004120
<i>Zygogynum bicolor</i> Tiegh.	Suh <i>et al.</i> 1993 / AY004118
<i>Zygogynum panchieri</i> (Baill.) Vink	Suh <i>et al.</i> 1993 / AY004119
CANELLACEAE	
<i>Capsicodendron denisii</i> (Schwake) Occhioni	Karol <i>et al.</i> 2000 / AY004132
<i>Canella winterana</i> (L.) Gaertn.	Suh <i>et al.</i> 1993 / LO3844
<i>Cinnamodendron ekmanii</i> Sleumer	Karol <i>et al.</i> 2000 / AY004133
<i>Cinnamosma madagascariensis</i> Danguy	Karol <i>et al.</i> 2000 / AY004131
<i>Pleodendron macranthum</i> (Baill.) Thiegh.	Karol <i>et al.</i> 2000 / AY004134
<i>Warburgia salutaris</i> (G. Bertol.) Chiov.	Karol <i>et al.</i> 2000 / AY004130

compared to previous results where indels were only mapped onto the phylogeny (Suh *et al.* 1993, Karol *et al.* 2000, Doust & Drinnan 2004). To estimate phylogeny within *Drimys*, a new matrix including five species of *Drimys* (including the two varieties of *D. winteri*) and two species of *Pseudowintera* Dandy as outgroup was constructed and analyzed using MP.

#### ANALYSES

MP analyses were conducted using PAUP\* 4.0 (Swofford 2000). The analysis were conducted for both matrices using a heuristic search option with 2000 iterations, retaining 5 trees for each replicate, and using the RAS + TBR + MULTREES option to save all possible minimum length trees. All characters

(base pair positions and indels) were considered as unordered and equally weighted (Fitch 1971). Relative support for the clades recovered was assessed via bootstrap analysis (Felsenstein 1985, Hills & Bull 1993) using 1000 replicates and 10 random addition sequences and TBR branch swapping.

#### RESULTS

The complete ITS sequences (without 5.8S) were 475 bp for *D. andina*, *D. confertifolia* and *D. angustifolia*, and 476 bp for each variety of *D. winteri*. Intraspecific variation was not found. Although all sequences are very similar among the species, the ITS1 region was more variable in length

than ITS2, ranging from 249 bp in *D. andina* to 252 bp in *D. winteri*. In the present work no variation was found among the varieties of *D. winteri*. However, we found few differences with those obtained by Suh *et al.* (1993). To align *Drimys* and *Pseudowintera* sequences, it was necessary to infer 18 indels. Nearly 53% of the variable sites among *Drimys* species are represented by indels. The complete data matrix (including Canellaceae and other Winteraceae taxa) had 739 characters, 112 were

indels, and 278 characters were parsimony informative. The *Drimys* and *Pseudowintera* matrix had 486 characters, 35 were parsimony informative, and 13 indels. Using the complete data matrix, parsimony analysis recovered six MP trees of 624 steps with a CI = 0.8221 and RI = 0.9 (477 steps, CI = 0.738 and RI = 0.9 with informative characters only). The cladogram (Fig. 2) shows all species of *Drimys* as a monophyletic group supported by a bootstrap value of 100%. In spite of an unresolved *Drimys*

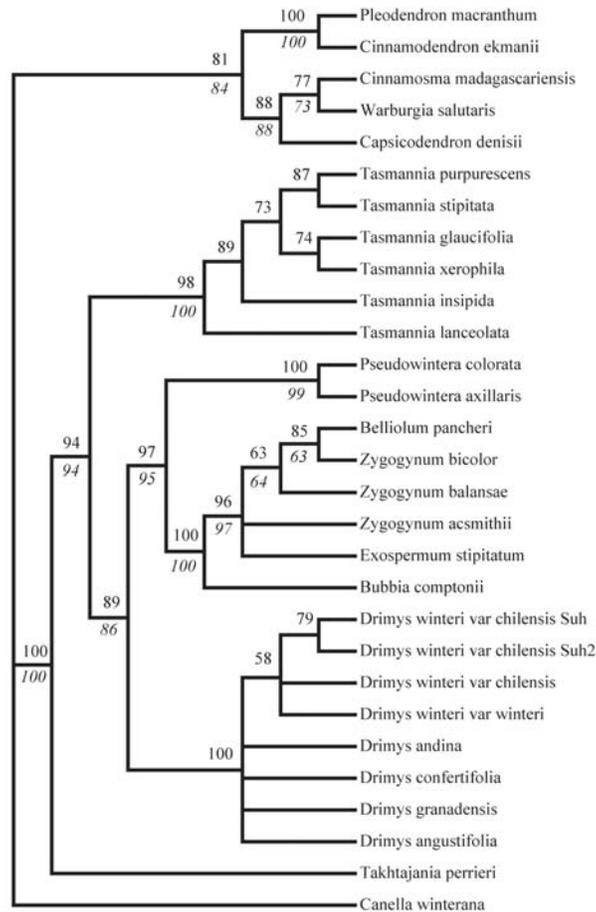


FIGURE 2. Strict consensus of 6 most parsimonious trees and generated from a combined ITS + indel matrix. Tree length = 624 steps (477 steps excluding uninformative characters), CI = 0.798 (0.738 excluding uninformative characters) and RI = 0.899 (0.899 excluding uninformative characters). Bootstrap support values greater than 50% are indicated above and below; values above the branches are estimated from our analyses whereas those below the branches are from Karol *et al.* (2000).

FIGURA 2. Consenso estricto obtenido de los 6 árboles más parsimoniosos y generado con los datos combinados del ITS y los indels. El largo del árbol = 624 pasos (477 excluyendo a los caracteres no informativos), CI = 0,798 (0,738 excluyendo a los caracteres no informativos) y un RI = 0,899 (0,899 excluyendo a los caracteres no informativos). Los números sobre y bajo las ramas corresponden a los índices de bootstrap calculados en este trabajo y los publicados por Karol *et al.* (2000), respectivamente.

branch, relationships among the remainder of Winteraceae are well resolved and consistent with those obtained by Suh *et al.* (1993), Karol *et al.* (2000) and Doust & Drinnan (2004).

The MP analysis of *Drimys*, considering *Pseudowintera* species as outgroup, recovered two most parsimonious trees of 67 steps with CI = 0.985, RI = 0.98 (48 steps, CI = 0.975, RI = 0.980, excluding uninformative characters, Fig. 3). Only bootstrap values

higher than 50% are shown above each branch. The only discrepancy between these trees involves the relationship among *D. andina* and *D. confertifolia* with the remaining species of *Drimys*. In Figure 3A, 54% of the characters supporting the branches are indels and in Figure 3B 60% of the characters are indels. Both cladograms show *D. andina* as the most divergent species. Although with low bootstrap support, *D. winteri* is represented as a monophyletic group.

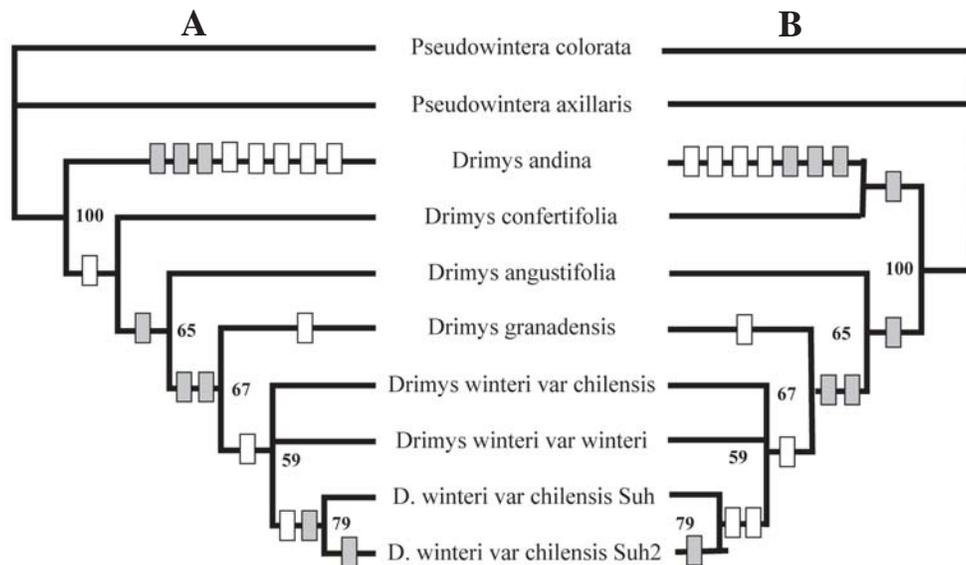


FIGURE 3. The two most parsimonious trees (A and B) obtained from ITS data plus indels. Tree length = 67 steps with CI = 0.985, RI = 0.98 (48 steps, CI = 0.975, RI = 0.980, excluding uninformative characters), with bootstrap values obtained from 50% majority rule tree. Bootstrap support values greater than 50% are indicated. Shaded squares represent indels whereas plain squares show molecular characters mapped on both trees.

FIGURA 3. Los dos árboles más parsimoniosos (A y B) obtenidos a partir del ITS más indels. Largo del árbol = 67 pasos con CI = 0,985, RI = 0,98 (48 pasos, CI = 0,975, RI = 0,980, excluyendo los caracteres no informativos). Los valores de soporte inferiores al 50% no fueron mostrados. Los cuadrados sombreados representan a las inserciones, mientras que cuadrados blancos muestran los caracteres moleculares mapeados en ambos árboles.

## DISCUSSION

The present work is the first attempt to study the relationships among species of *Drimys* from a molecular perspective. Previous studies have considered only one or two species in the context of a broad family-level phylogenetic analysis (Suh *et al.* 1993, Karol *et al.* 2000, Doust & Drinnan 2004). In this study we incorporated five species of *Drimys* as well as two varieties of *D. winteri* (var. *winteri*

and var. *chilensis*). As would be expected, the parsimony analysis of Winteraceae resulted in higher bootstrap support for clades when indels were included in the analysis. The phylogenetic hypothesis of Winteraceae obtained by Suh *et al.* (1993), Karol *et al.* (2000) and Doust & Drinnan (2004) were very similar, indicating that *Pseudowintera* is the most closely related genus to the common ancestor with *Drimys*. Although there were differences between our *D. winteri* sequences and

those of Suh *et al.* (1993), the varieties of this species form a monophyletic group.

ITS sequences of *Drimys* species are very similar, except for *D. andina*. Except for the latter species, this result is consistent with the high uniformity of morphological characters among the taxa (Smith 1943). *Drimys confertifolia* was expected to be closely allied to *D. winteri* based on the general biogeographic pattern that most species endemic to the Juan Fernandez Islands have their closest relatives in continental Chile (Skottsberg 1956, Stuessy *et al.* 1990, 2005, Ruiz *et al.* 2004). The relationships portrayed in the molecular phylogeny are somewhat unexpected because of the morphological similarity between *D. confertifolia* and *D. winteri*, and the aforementioned biogeographic relationships between continental Chile and the Juan Fernandez Islands. The early divergence of the island species in the phylogeny is also somewhat surprising and differs from other Juan Fernandez endemics such as the genera *Dendroseris* (Kim *et al.* 2007) and *Robinsonia* (Pelser *et al.* 2007), both of which are nested within otherwise continental genera. Since *D. confertifolia* diverged from a common ancestor of continental tree species within the last 4 millions year, the estimated age for Robinson Crusoe Island (Stuessy *et al.* 1984), this means that divergence among the majority of other contemporary species in *Drimys* occurred within the last four million years. It should be noted that our results showing an early divergence for *D. confertifolia* are concordant with the view of Smith (1943), who indicated that *D. confertifolia* is an ancestral species, based on its high number of ovules, a character that was considered by that author as primitive within the group.

Smith (1943) and Ehrendorfer *et al.* (1979) indicated that *D. andina* and *D. winteri* var. *winteri* may have originated from *D. winteri* var. *chilensis* where by both the Andes Mountain elevation and Pleistocene changes might have been important factors in the evolution of the continental Chilean species of *Drimys*. Palynologic records indicate "winteroid" pollen in Late Eocene from Argentinean Patagonia (Barreda 1997, Doyle 2000), although, in Chile the pollen record of *Drimys* dates only from the Quaternary (Heusser 1981, Heusser *et al.* 1999, Villagrán 2001) when the maximum elevation of Andean Mountains already existed (Solbrig *et al.* 1977). Thus, it is presumed that the species of *Drimys* distributed on the western side of the Andes

would be of more recent origin. Our results support this hypothesis because of the low ITS sequence divergence among western species.

What is problematical, however, is the high sequence divergence of *D. andina*, a species occurring west of the Andes (Fig. 1). A plausible explanation could be that *D. andina*, a shrubby species, was the first that diverged before the Quaternary, restricted to the high parts of the Andes, which reached their current elevation during the Pliocene (Solbrig 1976).

Ehrendorfer *et al.* (1979) stated that the Brazilian group (*D. angustifolia*, *D. brasiliensis* and *D. roraimensis*) was ancestral within the genus based on their analysis of morphological traits. Although, we were able to sequence only one species from Brazil, our results and the palynologic records agree with this hypothesis, indicating that *D. angustifolia* is the basalmost arborescent continental species. It would be highly desirable to have sequences of the other species from Brazil, *D. brasiliensis* and *D. roraimensis*, to test this hypothesis.

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