

## Phylogeographic Inferences Concerning Evolution of Brazilian *Passiflora actinia* and *P. elegans* (Passifloraceae) Based on ITS (nrDNA) Variation

ALINE P. LORENZ-LEMKE<sup>1</sup>, VALÉRIA C. MUSCHNER<sup>1</sup>, SANDRO L. BONATTO<sup>2</sup>,  
ARMANDO C. CERVI<sup>3</sup>, FRANCISCO M. SALZANO<sup>1</sup> and LORETA B. FREITAS<sup>1,\*</sup>

<sup>1</sup>Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501-970 Porto Alegre, RS, Brazil, <sup>2</sup>Centro de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, RS, Brazil and <sup>3</sup>Departamento de Botânica, Universidade Federal do Paraná, Caixa Postal 19031, 81531-970 Curitiba, PR, Brazil

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• **Background and Aims** *Passiflora actinia* and *P. elegans*, two markedly parapatric species, have their southern and northern distribution limits, respectively, in the most southern part of the Brazilian Atlantic Rain Forest. Despite the fact that they are classified in different taxonomic series, previous phylogenetic studies of this genus revealed a high genetic similarity between them. The aim of the present work was to analyse in more detail their geographical range in this region of overlap, to investigate intraspecific genetic variability and phylogeographic structure, and to search for possible hybrids.

• **Methods** Eighty-two localities were searched for these species, and nuclear internal transcribed spacer (ITS) sequences were investigated for 32 individuals of *P. actinia*, 20 of *P. elegans* and one putative interspecific hybrid. Plastid *trnL-trnF* and *psbA-trnH* were examined for 12 plants of each species and the putative hybrid.

• **Key Results** Both species showed a high level of intraspecific and intra-individual ITS variability. Network analysis revealed a north–south geographic gradient in their intra and interspecific relationships. Mismatch analyses suggested a recent population expansion of *P. elegans*. The plastid markers showed restricted variability but, together with the nuclear data, they contributed to the identification of an interspecific hybrid of intermediate morphology at the border of the distribution of these two species. Both genetic and morphological data indicate the absence of an extensive hybridization zone between these species.

• **Conclusions** Gene flow between lineages is the possible cause for the presence of different ITS sequences within a given plant, the absence of homogenization being due to the high degree of vegetative reproduction in the two species. Differentiation of *P. actinia* into geographic groups and the origin of *P. elegans* may have been influenced by the Atlantic Forest migration towards southern Brazil. The genetic pattern of the interspecific hybrid indicates that plastid inheritance in these species is at least sometimes paternal.

**Key words:** *Passiflora actinia*, *Passiflora elegans*, Passifloraceae, ITS, *trnL-trnF*, *psbA-trnH*, phylogeography, hybridization, quaternary climatic changes, Atlantic Forest, migration.

### INTRODUCTION

The southern portion of the Atlantic Forest is thought to have been formed by the migration of northern tropical elements (Rambo, 1951), the main event probably having occurred at the beginning of the Holocene (11 000–10 000 years BP) due to increasing temperature and humidity (Roth and Lorscheitter, 1993; Neves and Lorscheitter, 1995). The geographical range of animal and plant species that occur along the Brazilian coast was highly influenced by climatic oscillations in the Quaternary period, mainly due to several direct and indirect effects of changes in sea level that occurred during that period (Villwock and Tomazelli, 1995). These processes had a marked effect on the Atlantic Forest in the last 20 000 years and resulted in periods of expansion and contraction that may have led to fragmentation. The isolation of populations associated with forest fragments probably reduced the gene flow among them, but reproductive isolation does not always happen in such cases and hybrid zones may occur in situations of secondary contact. The analysis of nuclear and plastid DNA is being widely used to evaluate

such cases, allowing determination of the direction of the gene flow (Schaal *et al.*, 1998), etc.

The nuclear ribosomal DNA (nrDNA) of higher plants is organized in blocks in one or more chromosomal regions, each block consisting of hundreds to thousands of copies or paralogues (Buckler *et al.*, 1997). Mutations in these tandem repeats within an individual are generally homogenized through concerted evolution (Arnheim, 1983), but the mode and timing of this concerted evolution vary markedly among different groups of plants (Baldwin *et al.*, 1995; Mayer and Soltis, 1999). Generally the nrDNA intragenomic diversity is low, but some species show high heterogeneity in this region (Buckler *et al.*, 1997; Denduangboripant and Cronk, 2000). This can occur if the process is not sufficiently rapid to achieve homogenization of different copies (Campbell *et al.*, 1997; Zhang and Sang, 1999). After intraspecific or interspecific hybridization the different ITS copies (ribotypes) can evolve in different ways (Koch *et al.*, 2003). With more recent hybrids, direct sequencing can reveal an additive pattern (signal of two different nucleotides) in the material studied (Fuertes Aguilar and Nieto Feliner, 2003).

\* For correspondence. E-mail [loreta.freitas@ufrgs.br](mailto:loreta.freitas@ufrgs.br)

*Passiflora* is a highly diversified genus of Passifloraceae (passion-flowers), with many of the species being found in tropical America. About 130 *Passiflora* species occur in Brazil, with the highest number in the Amazon basin (Killip, 1938). The Brazilian state of Rio Grande do Sul, with its subtropical climate, harbours only 15 of these species (Mondin, 2001); among these, *P. elegans* is notable for being restricted to the state and adjacent regions. Phylogenetic analyses in *Passiflora* using the ITS region and the plastid *trnL-trnF* intergenic spacer revealed that *P. actinia* is closely related to *P. elegans* (Muschner *et al.*, 2003). Despite their high similarity, however, they had been previously classified in different taxonomic series (*P. actinia* in Simplicifoliae, *P. elegans* in Lobatae; Killip, 1938). This classification was based on leaf form, a highly variable trait that is subject to environmental influences in *Passiflora* (Benson *et al.*, 1976; MacDougal, 1994). These two species are parapatric: the northern limit of *P. elegans* is the southern limit of *P. actinia*, and there are no records of sympatry of these species, although both live in the same type of forested habitat. Unlike *P. elegans*, *P. actinia* is widely distributed, occurring from 18°S to 30°S along the eastern border of the Atlantic Forest from Espírito Santo to Rio Grande do Sul.

This study is a survey of the intraspecific variation in *P. actinia* and *P. elegans*, especially the contact area between them, but also in other regions of their distribution in southern Brazil. Nuclear ITS and plastid *trnL-trnF* and *psbA-trnH* markers were used to investigate their relationships and possible hybridization events. The patterns observed were examined taking into consideration the processes of separation and retraction of the Atlantic Forest in the region, and their eventual connections with the evolutionary relationships of these two taxa.

## MATERIALS AND METHODS

### *Samples and DNA isolation*

Leaf material from 32 plants of *P. actinia*, 20 from *P. elegans* and one from a putative hybrid between *P. actinia* and *P. elegans* were obtained at 41 collecting sites (Table 1 and Fig. 1). For the plastid studies, 12 *P. actinia*, 11 *P. elegans* and the hybrid were chosen in a way that would adequately cover the study area. Voucher specimens were deposited in the ICN Herbarium, Botany Department, Biosciences Institute, Federal University of Rio Grande do Sul. Total DNA was extracted from young leaves dried in silica gel, using the method of Roy *et al.* (1992) with a few adaptations.

### *Polymerase chain reaction (PCR) amplification and sequencing*

The segments chosen for study were: (a) the internal transcribed spacer (ITS) region of nrDNA, using primers and amplification conditions described by Desfeux and Lejeune (1996); and (b) plastid *trnL-trnF* and *psbA-trnH* intergenic spacers, employing, respectively, the primers and amplification conditions indicated by Taberlet *et al.* (1991) (primers e and f) and Sang *et al.* (1997). Dimethyl sulfoxide

(DMSO) at 10 % was used in the ITS PCR, to exclude the presence of silenced alleles (Buckler *et al.*, 1997; Fuertes Aguilar *et al.*, 1999). The amplified material was purified with shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences, Amersham, Bucks., UK) and the strands directly sequenced using the BigDye Terminator Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI Prism 310 (Perkin Elmer, Wellesley, USA) genetic analyser.

### *Sequence analyses*

The GenBank numbers of *P. actinia* and *P. elegans* sequences are given in Table 1. For ITS, a site was classified as heterozygous when more than one peak was present in the electropherogram and the weakest signal reached at least 25 % of the strength of the strongest signal (Fuertes Aguilar *et al.*, 1999; Fuertes Aguilar and Nieto Feliner, 2003). To minimize the inclusion of bad reads as polymorphisms, as suggested by these authors, the restriction was added that double peaks had to occur on the same position in both strands. The sequences were aligned in the ClustalX1.81 program (Thompson *et al.*, 1997), with manual corrections using the GeneDoc program (<http://www.psc.edu/biomed/genedoc>). The variable sites and nucleotide diversity were estimated using the MEGA version 2.1 program (Kumar *et al.*, 2001). For heterozygous individuals, ITS sequence types were estimated using the PHASE program, version 1.0 (Stephens *et al.*, 2001), with manual resolution of the few not determined by the program. Relationships among sequences were inferred using median-joining networks ( $\epsilon = 0$ ; Bandelt *et al.*, 1999) with the NETWORK program, version 3.1.1.1, available at (<http://www.fluxus-engineering.com>). Tajima's D (Tajima, 1989), Fu and Li's  $D^*$  (Fu and Li, 1993) and observed and expected mismatch distribution graphics, were conducted using the DNAsp 3.99 program (Rozas and Rozas, 1999). *Passiflora sidaefolia* sequences were used as the outgroup.

### *Seed viability*

The seed viability of a possible *P. actinia* × *P. elegans* hybrid was investigated by the tetrazolium test (Bonner, 1986). Twenty seeds from three fruits of plant E56 (the putative hybrid) and E30 (which acted as the control) were investigated. The seeds were placed in distilled water at 30 °C for 24 h, longitudinally cut, soaked in a 0.5 % (w/v) 2,3,5 triphenyl tetrazolium chloride solution, and stored at 30 °C until the embryo acquired a reddish coloration (maximum time 48 h).

## RESULTS

### *Geographical range of the species*

Field trips were taken to 81 localities in the Brazilian states of Rio Grande do Sul (RS), Santa Catarina (SC) and Paraná (PR). Localities where *P. actinia* and *P. elegans* were found are indicated in Fig. 1. Our collections confirmed previous records that the southernmost geographic range of *P. actinia*

TABLE 1. Characterization of the *P. actinia* and *P. elegans* specimens studied

Species and specimen code	Geographical location*		Collector's name or code	ITS sequences	GenBank accessions			
	Locality	Geographical coordinates			ITS 1	ITS 2	<i>trnL-trnF</i>	<i>psbA-trnH</i>
<i>Passiflora actinia</i>								
A3	Três Cachoeiras, RS	29°24'S, 50°06'W	C. Mondin	H8A/H8A	AY219247	AY219271	–	–
A8	São Francisco de Paula, RS	29°26'S, 50°36'W	APL 014	H7A/H7A	AY219248	AY219272	AY219312	AY219288
A10	Serra do Pinto, RS	29°21'S, 50°10'W	APL 016	H9A/H9A	AY219249	AY219273	AY219313	AY219289
A12	Praia Grande, SC	29°10'S, 49°56'W	APL 019	H9A/H13A	AY219250	AY219274	AY219314	AY219290
A15	Praia Grande, SC	29°10'S, 49°56'W	APL 022	H10A/H16A	AY219246	AY219270	–	–
A16	Jd. América, Curitiba, PR	25°30'S, 49°18'W	A. Cervi	H18A/H18A	AY219240	AY219264	–	–
A17	Centro Politécnico, Curitiba, PR	25°26'S, 49°14'W	A. Cervi	H18A/H18A	AY542629	AY542658	AY219315	AY219291
A18	Centro Politécnico, Curitiba, PR	25°26'S, 49°14'W	A. Cervi	H18A/H18A	AY542630	AY542659	–	–
A19	Centro Politécnico, Curitiba, PR	25°26'S, 49°14'W	A. Cervi	H11A/H17A	AY219244	AY219268	–	–
A20	Jd. Botânico, Curitiba, PR	25°26'S, 49°14'W	A. Cervi	H21A/H21A	AY219243	AY219267	–	–
A22	FLONA, São Francisco de Paula, RS	29°23'S, 50°22'W	APL 030	H10A/H16A	AY542640	AY542669	–	–
A23	Serra do Umbu, RS	29°29'S, 50°19'W	APL 032	H7A/H14A	AY219255	AY219279	AY219316	AY219292
A24	J. Velho, São Francisco de Paula, RS	29°28'S, 50°40'W	PASS 037	H10A/H16A	AY542641	AY542670	–	–
A32	D. Pedro de Alcântara, RS	29°22'S, 49°50'W	PASS 059	H7A/H15A	AY542642	AY542671	AY219317	AY219293
A37	Serra da Boa Vista, RS	29°34'S, 50°20'W	APL 055	H7A/H15A	AY219252	AY219276	AY219318	AY219294
A39	Barra do Ouro, RS	29°30'S, 50°16'W	APL 062	H7A/H9A	AY219254	AY219278	AY219319	AY219295
A42	Osório, RS	29°48'S, 50°18'W	PASS 069	H7A/H13A	AY219253	AY219277	AY219320	AY219296
A47	Taquara, RS	29°43'S, 50°42'W	PASS 074	H7A/H15A	AY542643	AY542672	AY219321	AY219297
A50	Canela, RS	29°21'S, 50°48'W	APL 105	H9A/H14A	AY219251	AY219275	AY219322	AY219298
A56	Reserva Biol. da Serra Geral, RS	29°34'S, 50°12'W	APL 115	H7A/H15A	AY542644	AY542673	AY219323	AY219299
A70	Urubici, SC	28°06'S, 49°36'W	A. Cervi	H12A/H18A	AY219245	AY219269	–	–
A88	Centro Politécnico, Curitiba, PR	25°26'S, 49°14'W	APL 183	H18A/H18A	AY542631	AY542660	–	–
A89	B. Gutierrez, Curitiba, PR	25°24'S, 49°17'W	APL 184	H18A/H18A	AY542632	AY542661	–	–
A90	B. Gutierrez, Curitiba, PR	25°24'S, 49°17'W	APL 185	H18A/H20A	AY219241	AY219265	–	–
A92	B. Alemão, Curitiba, PR	25°24'S, 49°17'W	APL 187	H18A/H18A	AY542633	AY542662	–	–
A93	B. Alemão, Curitiba, PR	25°24'S, 49°17'W	APL 188	H18A/H18A	AY542634	AY542663	–	–
A98	São José dos Pinhais, PR	25°32'S, 49°13'W	APL 195	H18A/H18A	AY542635	AY542664	–	–
A110	São José dos Pinhais, PR	25°32'S, 49°13'W	APL 208	H18A/H18A	AY542636	AY542665	–	–
A113	B. Cascatinha, Curitiba, PR	25°24'S, 49°18'W	APL 212	H18A/H18A	AY542637	AY542666	–	–
A115	Pq. Barigüi, Curitiba, PR	25°25'S, 49°18'W	APL 214	H18A/H18A	AY542638	AY542667	–	–
A123	Pq. Passatúna, Curitiba, PR	25°27'S, 49°22'W	APL 222	H18A/H18A	AY542639	AY542668	–	–
A126	B. Zaninelli, Curitiba, PR	25°23'S, 49°16'W	APL 225	H18A/H19A	AY219242	AY219266	–	–
<i>Passiflora elegans</i>								
E5	Morro Santana, Porto Alegre, RS	30°04'S, 51°07'W	PASS 010	H3E/H6E	AY219258	AY219282	–	–
E12	Caçapava do Sul, RS	30°33'S, 53°32'W	PASS 026	H6E/H6E	AY542645	AY542674	AY219324	AY219300
E13	Caçapava do Sul, RS	30°33'S, 53°32'W	PASS 027	H4E/H6E	AY219262	AY219286	–	–
E16	Portão, RS	29°42'S, 51°14'W	PASS 033	H1E/H3E	AY219259	AY219283	AY219325	AY219301
E20	Ponta do Cego, Porto Alegre, RS	30°15'S, 51°06'W	PASS 063	H1E/H1E	AY219260	AY219284	AY219326	AY219302
E22	Pelotas, RS	31°48'S, 52°24'W	APL 068	H1E/H1E	AY542655	AY542684	AY219327	AY219303
E26	Guaíba, RS	30°04'S, 51°24'W	APL 072	H3E/H6E	AY542650	AY542679	AY219328	AY219304
E29	Cachoeira do Sul, RS	30°16'S, 52°57'W	APL 078	H3E/H6E	AY542651	AY542680	AY219329	AY219305
E30	Santo Antônio da Patrulha, RS	29°50'S, 50°30'W	PASS 116	H1E/H3E	AY542654	AY542683	AY219330	AY219306
E38	Lavras do Sul, RS	30°52'S, 53°43'W	APL 093	H3E/H6E	AY542652	AY542681	AY219331	AY219307
E44	Paverama, RS	29°36'S, 51°48'W	PASS 097	H2E/H6E	AY219261	AY219285	AY219332	AY219308
E45	Paverama, RS	29°36'S, 51°48'W	PASS 098	H2E/H6E	AY542657	AY542686	–	–
E48	Lagoa dos Três Cantos, RS	28°33'S, 52°51'W	PASS 103	H3E/H6E	AY542653	AY542682	AY219333	AY219309
E49	São Miguel das Missões, RS	28°32'S, 54°33'W	PASS 105	H1E/H1E	AY542656	AY542685	AY219334	AY219310
E50	Espumoso, RS	28°49'S, 52°40'W	PASS 110	H6E/H6E	AY542646	AY542675	–	–
E51	Lageado, RS	29°20'S, 52°05'W	PASS 111	H5E/H5E	AY219256	AY219280	–	–
E53	Lageado, RS	29°20'S, 52°05'W	PASS 113	H6E/H6E	AY219257	AY219281	–	–
E55	Lageado, RS	29°20'S, 52°05'W	PASS 115	H6E/H6E	AY542647	AY542676	–	–
E56†	Santo Antônio da Patrulha, RS	29°50'S, 50°30'W	PASS 118	H15A/H3E	AY219263	AY219287	AY219335	AY219311
E72	BR290, RS	30°09'S, 52°08'W	PASS 214	H6E/H6E	AY542648	AY542677	–	–
E75	BR386, RS	29°33'S, 51°53'W	PASS 267	H6E/H6E	AY542649	AY542678	–	–

\* The abbreviations PR, RS and SC indicate the Brazilian states of Paraná, Rio Grande do Sul and Santa Catarina, respectively.

† Interspecific hybrid between *Passiflora actinia* and *P. elegans*.

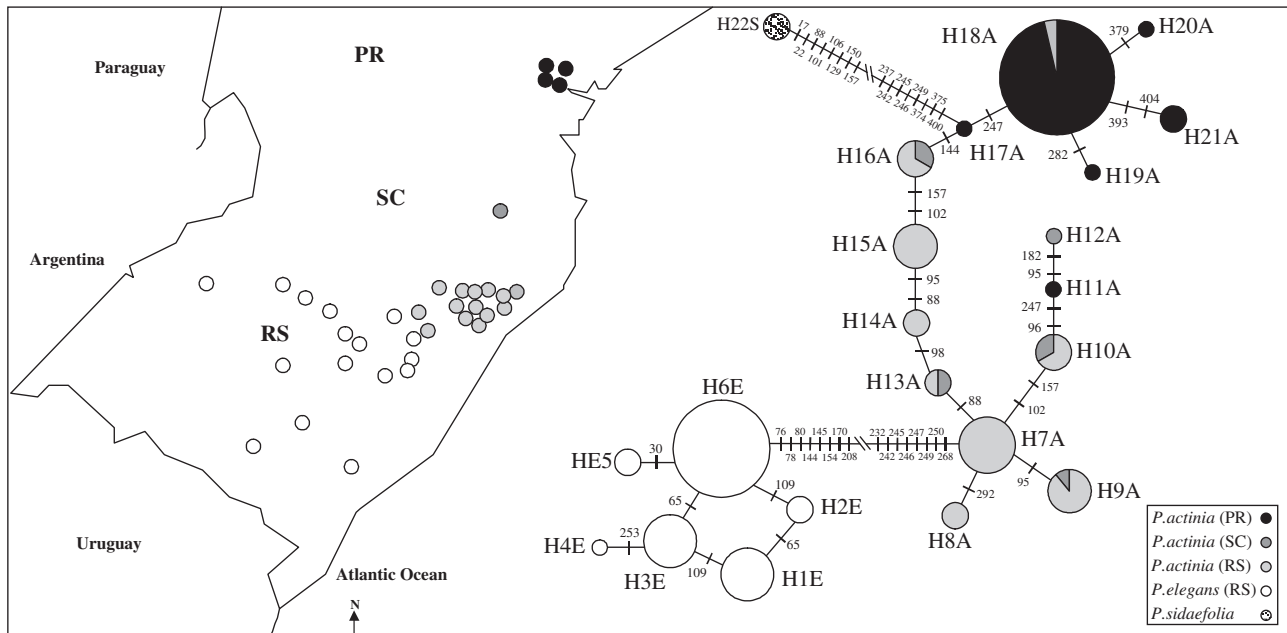


FIG. 1. Left, geographical ranges and collecting places of *Passiflora actinia* (black and light- and dark-shaded circles) and *P. elegans* (white circles). The abbreviations PR, SC, and RS indicate the Brazilian states of Paraná, Santa Catarina and Rio Grande do Sul, respectively. Right, median-joining network connecting the different ITS sequences. The diameters of the circles indicate their frequencies, and the degree of shading their origin: white, *P. elegans*, Rio Grande do Sul; light shading, *P. actinia*, Rio Grande do Sul; dark shading, *P. actinia*, Santa Catarina; black, *P. actinia*, Paraná; dotted, *P. sidaefolia*, used as an outgroup. Numbers between the circles indicate the sites where changes occurred, separating the sequences.

is the north-east region of Rio Grande do Sul, being restricted to Atlantic Forest areas, and that *P. elegans* occurs in the riparian vegetation of the interior of Rio Grande do Sul. Especially in *P. elegans*, large distances were observed between the sampled populations, and it is hypothesized that this isolation may be related to the high fragmentation of the forest formation with which this species is associated. Populations of both species were composed of a small number of large plants with many branches, making it difficult to ensure that samples came from different individuals.

#### ITS variation

The ITS 1 and ITS 2 sequences varied between 227 and 228 bp, and 171 and 175 bp in *P. actinia* and *P. elegans*, respectively. After alignment the two spacers were analysed together, totalling a matrix of 404 characters (average of 66.7 % GC content). Thirty-two polymorphic sites were found, of which 14 corresponded to fixed interspecies variation, 14 to intraspecific differences in *P. actinia* and four to intraspecific variability in *P. elegans*. A large part of this variability was due to heterozygous sites in some individuals (57 % of the polymorphic sites in *P. actinia*, and 75 % in *P. elegans* showed this type of variation). Six sequence types were identified in *P. elegans* (H1E–H6E) and 15 in *P. actinia* (H7A–H21A; Table 2). Plant E56 was identified as a hybrid (see below) due to its morphological appearance and the occurrence of sequences from both species (H3E/H15A). Nucleotide diversity values were 0.006 for *P. actinia* and 0.002 for *P. elegans*.

The median-joining network (Fig. 1) clearly showed a north–south direction in the inter- and intraspecific

relationships. A clear separation exists between the northern and southern sequences in *P. actinia*, but there is a series of intermediate sequence types. All *P. elegans* sequences were consistently associated with those obtained from southern *P. actinia* plants. In *P. elegans*, however, it was not possible to identify a relationship between sequences and the geographical origin of the plant. Tajima's *D* (*P. actinia*: 1.077;  $P > 0.10$ , *P. elegans*: 0.689;  $P > 0.10$ ) and Fu and Li's *D*\* (*P. actinia*:  $-0.966$ ;  $P > 0.10$ , *P. elegans*:  $-0.357$ ;  $P > 0.10$ ) neutrality tests showed non-significant values. A single wave in the mismatch distribution—a histogram of genetic differences between pairs of individuals within a sample—with a peak at about one difference was found among *P. elegans* plants (Fig. 2) indicating a recent population expansion. The *P. sidaefolia* (AY102353/AY102373) sequence used as an outgroup connected to the network through the sequences of northern individuals of *P. actinia*. A reticulation was found between H1E, H2E, H3E and H6E, due to homoplastic substitutions at sites 65 and 109 or to recombination between some copies (Fig. 1).

#### Plastid DNA markers

The results are displayed in Table 1. The *trnL-trnF* and *psbA-trnH* spacers ranged in size from 288 to 294 bp and from 309 to 311 bp, respectively (averages, respectively, of 36.1 and 28.9 % GC content). For the *trnL-trnF* spacer, two differences were found, a 6-bp indel (TAAGAT, sites 265–270) present in *P. actinia* and absent in *P. elegans*; and a C (*P. actinia*)/A (*P. elegans*) change at site 196. In the *psbA-trnH* spacer a substitution at position 59, T (*P. actinia*)/G (*P. elegans*), and a region characterized by T repetitions



TABLE 2. Sequences identified in ITS regions 1 and 2 of the Passiflora plants investigated

Sequence type	Nucleotide position																																								No. observed	
	ITS 1																				ITS 2																					
H1E	-	A	T	C	T	C	T	A	G	G	-	T	A	G	C	C	-	G	-	C	C	C	T	G	-	G	G	C	-	C	C	C	T	G	-	G	G	C	8			
H2E	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
H3E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8	
H4E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	
H5E	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2		
H6E	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	20		
H8A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	T	.	.	.	2			
H7A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	9			
H9A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	5			
H10A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	3			
H11A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	1			
H12A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	1			
H13A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	2			
H14A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	2			
H15A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	5			
H16A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	3			
H17A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	1			
H18A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	27			
H19A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	1			
H20A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	1			
H21A	.	C	C	T	C	.	.	.	.	A	A	.	G	A	A	C	C	C	C	C	C	C	C	T	A	.	A	.	A	.	A	.	A	.	.	.	.	.	2			

Dots indicate agreement with the reference sequence (H1E) and dashes deletions at the indicated positions.

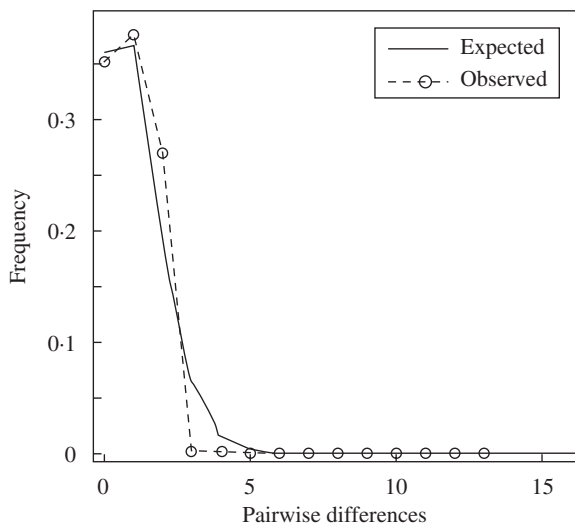


FIG. 2. Mismatch distribution histogram indicating the observed and expected numbers of pairwise differences in ITS sequences between *P. elegans* plants. The numbers of pairwise differences are given on the horizontal axis and their frequencies on the vertical axis.

(sites 163–172) were found. The latter varied both between species and among plants. All *P. actinia* plants had eight Ts. In *P. elegans* plants E16, E29 and E44 had 10 Ts, whereas the others all had nine. Plant E56 of *P. elegans* showed sequences in both plastid spacers identical to those of *P. actinia*.

#### A natural hybrid between *P. actinia* and *P. elegans*

Although the two species were not found in sympatry, a population of *P. elegans* was found only 9 km away from a *P. actinia* population in Santo Antonio da Patrulha county (29°50'S; 50°30'W). At this site a member of the *P. elegans* population (E56) was morphologically quite distinct from the others, showing a clearly intermediate phenotype, especially in relation to flower size and leaf shape. The main morphological distinctive characteristics of the two species are that in *P. actinia* the flowers are larger and the leaves are unlobed, while the leaves of *P. elegans* are slightly trilobed. In E56 leaves showed various patterns of lobation and the flowers were intermediate in size. All three molecular markers employed support the hypothesis that E56 is a hybrid between *P. actinia* and *P. elegans*, as suggested by its intermediate morphology and geographic localization (at the border of the distributions of the two species). The tetrazolium test indicated that seed viability in the hybrid was normal.

## DISCUSSION

### ITS genetic diversity

Paralogues of nrDNA can become pseudogenes when inactivated or when a single copy is transported to a different genomic region. These pseudogenes can accumulate random substitutions at high rates, reducing the stability of the secondary structure (Buckler and Holtsford, 1996;

Koch *et al.*, 2003). It is unlikely, however, that the sequences analysed in this study are pseudogenes, since: (a) DMSO was added to the PCR process, diminishing the possibility of amplification of less stable forms (Buckler *et al.*, 1997, Fuertes Aguilar *et al.*, 1999); (b) the transition/transversion rates and the GC contents are not different from the pattern generally found in the genus (Muschner *et al.*, 2003); and (c) several of the parental copies of the heterozygous individuals were identified.

The high level of ITS polymorphism found here indicates that the process of concerted evolution is not fast enough to homogenize the region. This process acts through recombination or gene conversion (Campbell *et al.*, 1997). Inter-chromosomal exchanges can be facilitated by the terminal location of the nrDNA *loci*, as occur in *Gossypium*, *Paeonia* and *Thinopyrum* (Wendel *et al.*, 1995; Zhang and Sang, 1999; Li and Zhang, 2002) but in the present case this does not seem to be the case, since Melo and Guerra (2003) mapped the 45S sites, where the ITS region is located, in subterminal positions in *P. actinia* and *P. elegans*.

Vegetative reproduction may extend generation time, and this may slow the speed with which concerted evolution/homogenization through recombination can occur (Sang *et al.*, 1995; Buckler IV and Holtsford, 1996). Vegetative reproduction is common in *P. actinia* and *P. elegans*. Plants of these species produce many shoots from roots that develop into complete plants; this fact, coupled with recent gene flow between divergent lineages, may explain the ITS polymorphism observed here. The process, however, was not important enough to obscure the interspecific relationships observed in the genus (Muschner *et al.*, 2003).

### Relationships of *P. actinia* and *P. elegans* and the Quaternary palaeoclimatic history of Rio Grande do Sul

It is clear from the molecular data presented here, as well as other molecular analyses performed by Muschner *et al.* (2003), that *P. actinia* and *P. elegans* are closely related, possible sister species. Additionally, *P. actinia* and *P. elegans* have the same chromosome number ( $2n = 18$ ; Melo and Guerra, 2003), show extremely similar flower morphology and blossom at the same period of the year. In relation to the scenario for their divergence, one hypothesis would postulate that *P. elegans* diverged from *P. actinia* before the occurrence of the intraspecific differentiation found today in the latter species. The other scenario would imply that *P. elegans* diverged from southern populations of *P. actinia* after the latter separated from the northern groups. However, the phylogenetic connection of *P. sidaefolia* with the northern clade of *P. actinia* (Fig. 1) suggests a clear direction on the divergence of these species, and summing up all genetic, geographic and morphological evidence, the proposition that *P. elegans* diverged from an already differentiated southern group of *P. actinia* is favoured.

*Passiflora actinia* shows a high ITS diversity that seems to be geographically structured (Fig. 1), suggesting relatively old populations (Avise, 2000). Conversely, *P. elegans* shows lower variability and this variation is not geographically structured. The high frequency of sequence H6E,

its central position in the network, and its geographic distribution suggest that it may be the plesiomorphic sequence in *P. elegans*. The left skewed single peak at about one difference in the mismatch distribution (Fig. 2), on the other hand, is an indication that this species went through a severe bottleneck followed by a recent expansion in size and possibly range (Rogers and Harpending, 1992).

Influence of the Pleistocene glaciations on the distribution and genetic diversity of plants has been much studied by molecular markers (Gielly *et al.*, 2001; Zhang *et al.*, 2001), but these studies were mainly limited to the northern hemisphere, with very few relating to tropical forests and even fewer to the species-rich South American Atlantic Forest. Pollen studies performed by Roth and Lorscheitter (1993) and Neves and Lorscheitter (1995) suggest that the central plateau and coastal plains of Rio Grande do Sul had a cold, semiarid climate in the last Pleistocene glacial stage (23 000–11 000 years BP). Open vegetation prevailed and forests were restricted to ecological refuges. The two main events of Atlantic Forest expansion from northern locations into Rio Grande do Sul are related to periods of significant climatic improvement, with rising temperatures and humidity, one at the beginning of the Holocene (11 000–10 000 years BP) and another around 6700 years BP (Lorscheitter, 1997). The data suggest that these migrations may have occurred in a fragmentary way, leading to spatial isolation of populations. The phylogeographic scenarios found here for both *P. actinia* and *P. elegans* seems to agree closely with the climatic and biogeographic scenarios described above for the Atlantic Forest in southern Brazil.

The presence of a viable hybrid at the place where the two species are geographically closest indicates secondary contact but absence of a hybrid zone, probably due to their spatial segregation, possibly influenced by external environmental agents, such as humidity, soil type, altitude, rainfall and other ecological factors (as described for other species by Fritsche and Kaltz, 2000; Gielly *et al.*, 2001).

#### *Genetic characterization of the hybrid*

Interspecific hybridization is a widespread phenomenon in plants (Rieseberg, 1997). Natural hybrids are frequent in *Passiflora* (Vanderplank, 1996), but had not been previously described between *P. actinia* and *P. elegans*. In the present study, a single hybrid plant was identified due to its intermediate floral and vegetative morphology, a finding that was afterwards confirmed by the genetic markers, with the nuclear nucleotide sites that differentiate *P. actinia* and *P. elegans* showing an additive pattern in the hybrid. The plastid markers indicated that *P. actinia* was the donor species of the plastid DNA in this hybrid. It was found in a *P. elegans* population, the closest *P. actinia* group occurring 9 km apart. Therefore, it is more likely that this hybrid occurred as a result of a single pollen dispersion and that plastid transmission in these species may be paternal, in contrast to the normal maternal inheritance in flowering plants. The only species of *Passiflora* considered (*P. edulis*) in a previous study of organelle inheritance in plants (Harris and Ingram, 1991) showed evidence, through epifluorescence microscopic studies, of paternal plastid

inheritance. In addition, molecular markers have indicated that *Turnera ulmifolia* (also Passifloraceae; Angiosperm Phylogeny Group, 2003) shows a mixed, but paternally biased pattern of plastid inheritance (Shore and Triassi, 1998), indicating with the present results that paternal plastid inheritance may be of more widespread occurrence in this group of plants. Mitochondrial DNA in the hybrid documented here is, however, of maternal (*P. elegans*) origin (V. C. Muschner, unpubl. res.).

## CONCLUSIONS

The present work confirmed previous records that the southernmost geographical range of *P. actinia* is the Atlantic Forest area situated in the north-eastern region of the Brazilian state of Rio Grande do Sul, whereas *P. elegans* occurs in the riparian vegetation of the interior of that state. The variability found in *P. actinia* is structured along a north–south gradient, whereas *P. elegans* has experienced a recent population bottleneck followed by an expansion. The recent demographic histories of both species were strongly influenced by the climatic changes that occurred in this area during the Quaternary period. A natural hybrid was found at the border of the distribution of these two species, and its genetic constitution suggests that plastid inheritance is paternal in these species.

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## LITERATURE CITED

- Angiosperm Phylogeny Group.** 2003. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* **141**: 399–436.
- Arnheim N.** 1983. Concerted evolution of multigene families. In: Nei M, Koehn RK, eds. *Evolution of genes and proteins*. Sunderland: Sinauer, 38–61.
- Avise JC.** 2000. *Phylogeography: the history and formation of species*. Cambridge: Harvard University Press.
- Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ.** 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. [http://www.ncbi.nlm.nih.gov/80/entrez/query.fcgi?cmd=Retrieve&db=journals&list\\_uids=20275&dopt=full](http://www.ncbi.nlm.nih.gov/80/entrez/query.fcgi?cmd=Retrieve&db=journals&list_uids=20275&dopt=full) **82**: 247–277.
- Bandelt H-J, Forster P, Röhl A.** 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.
- Benson WW, Brown Jr KS, Gilbert LE.** 1976. Coevolution of plants and herbivores: passion flower butterflies. *Evolution* **29**: 659–680.

- Bonner FT. 1986. Measurement of seed vigor for loblolly (*Pinus taeda*) and slash pines (*Pinus elliottii*). *Forest Science* 32: 170–178.
- Buckler ES IV, Holsford TP. 1996. *Zea* ribosomal repeat evolution and substitution patterns. *Molecular Biology and Evolution* 13: 623–632.
- Buckler ES IV, Ippolito A, Holsford TP. 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics* 145: 821–832.
- Campbell CS, Wojciechowski MF, Baldwin BG, Lawrence AA, Donoghue MJ. 1997. Persistent nuclear ribosomal DNA sequence polymorphism in the *Amelanchier* agamic complex (Rosaceae). *Molecular Biology and Evolution* 14: 81–90.
- Denduangboripant J, Cronk QCB. 2000. High intraindividual variation in internal transcribed spacer sequences in *Aeschynanthus* (Gesneriaceae): implications for phylogenetics. *Proceedings of the Royal Society of London* 267: 1407–1415.
- Desfeux C, Lejeune, B. 1996. Systematics of Euromediterranean *Silene* (Caryophyllaceae): evidence from a phylogenetic analysis using ITS sequences. *Comptes Rendus de l'Academie des Sciences de Paris* 319: 351–358.
- Fritsche F, Kaltz O. 2000. Is the *Prunella* (Lamiaceae) hybrid zone structured by an environmental gradient? Evidence from a reciprocal transplant experiment. *American Journal of Botany* 87: 995–1003.
- Fu YX, Li WH. 1993. Statistical tests of neutrality of mutations. *Genetics* 133: 693–709.
- Fuertes Aguilar J, Nieto Feliner G. 2003. Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). *Molecular Phylogenetics and Evolution* 28: 430–447.
- Fuertes Aguilar J, Rosselló JA, Nieto Feliner G. 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). *Molecular Ecology* 8: 1341–1346.
- Gielly L, Debussche M, Thompson JD. 2001. Geographic isolation and evolution of Mediterranean endemic *Cyclamen*: insights from chloroplast *trnL* (UAA) intron sequence variation. *Plant Systematics and Evolution* 230: 75–88.
- Harris SA, Ingram R. 1991. Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. *Taxon* 40: 393–412.
- Killip EP. 1938. The American species of Passifloraceae. *Botanical Series, Field Museum of Natural History* 19: 1–613.
- Koch MA, Dobes C, Mitchell-Olds T. 2003. Multiple hybrid formation in natural populations: concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricata* (Brassicaceae). *Molecular Biology and Evolution* 20: 338–350.
- Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA. Program available on the internet (<http://www.megasoftware.net>).
- Li D, Zhang X. 2002. Physical localization of the 18S-5.8S-26S rDNA and sequence analysis of ITS regions in *Thinopyrum ponticum* (Poaceae: Triticeae): implications for concerted evolution. *Annals of Botany* 90: 445–452.
- Lorscheitter ML. 1997. Paleoambientes do sul do Brasil no Quaternário através da palinologia: revisão dos resultados obtidos. *Revista Universidade de Guarulhos, Geociências* 2: 197–199.
- MacDougal JM. 1994. Revision of *Passiflora*, subgenus Decaloba, Section Pseudodysosmia (Passifloraceae). *Systematic Botany Monographs* 41: 1–146.
- Mayer MS, Soltis PS. 1999. Intraspecific phylogeny analysis using ITS sequences: insights from studies of the *Streptanthus glandulosus* complex (Cruciferae). *Systematic Botany* 24: 47–61.
- Melo NF de, Guerra M. 2003. Variability of the 5S and 45S rDNA sites in *Passiflora* L. species with distinct base chromosome numbers. *Annals of Botany* 92: 309–316.
- Mondin CA. 2001. *Passiflora organensis* Gardner (Passifloraceae), primeira citação de ocorrência para o Rio Grande do Sul. *Pesquisas-Botânica* 51: 147–150.
- Muschner VC, Lorenz AP, Cervi AC, Bonatto SL, Souza-Chies TT, Salzano FM, Freitas LB. 2003. A first molecular phylogenetic analysis in *Passiflora* (Passifloraceae). *American Journal of Botany* 90: 1229–1238.
- Neves PCF, Lorscheitter ML. 1995. Upper Quaternary paleoenvironments in the northern coastal plain of Rio Grande do Sul, Brazil. *Quaternary South American Antarctic Peninsula* 9: 39–67.
- Rambo B. 1951. A imigração da selva higrófila no Rio Grande do Sul. *Sellowia* 3: 55–91.
- Rieseberg LH. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* 28: 359–389.
- Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* 9: 552–569.
- Roth L, Lorscheitter ML. 1993. Palynology of a bog in Parque Nacional de Aparados da Serra, east plateau of Rio Grande do Sul, Brazil. *Quaternary South American Antarctic Peninsula* 8: 39–69.
- Roy A, Frascaria N, Mackay J, Bousquet J. 1992. Segregating random amplified polymorphic DNAs (RAPDs) in *Betula alleghaniensis*. *Theoretical and Applied Genetics* 85: 173–180.
- Rozas J, Rozas R. 1999. DnaSP, version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15: 174–175.
- Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences of the USA* 92: 6813–6817.
- Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120–1136.
- Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA. 1998. Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* 7: 465–474.
- Shore JS, Triassi M. 1998. Paternally biased cpDNA inheritance in *Turnera ulmifolia* (Turneraceae). *American Journal of Botany* 85: 328–332.
- Stephens M, Smith NJ, Donnelly P. 2001. A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics* 68: 978–989.
- Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.
- Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123: 585–595.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882. Program available on the internet (<ftp://ftp-igbmc.u-strasbg.fr/pub/cluster/>).
- Vanderplank J. 1996. *Passion flowers, 2nd edn*. Cambridge: Massachusetts Institute of Technology Press.
- Villwock JA, Tomazelli LJ. 1995. Geologia costeira do Rio Grande do Sul. *Universidade Federal do Rio Grande do Sul, Notas Técnicas* 8: 1–45.
- Wendel JF, Schnabel A, Seelanan T. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). *Proceedings of the National Academy of Sciences of the USA* 92: 280–284.
- Zhang D, Sang T. 1999. Physical mapping of ribosomal RNA genes in peonies (*Paeonia*, Paeoniaceae) by fluorescent *in situ* hybridization: implications for phylogeny and concerted evolution. *American Journal of Botany* 86: 735–740.
- Zhang L-B, Comes HP, Kadereit JW. 2001. Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *American Journal of Botany* 88: 2331–2345.