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A Phylogeographic Split in *Buxus balearica* (Buxaceae) as Evidenced by Nuclear Ribosomal Markers: When ITS Paralogues Are Welcome

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Abstract. Sequences from the ribosomal nuclear internal transcribed spacers (ITS) have been widely used to infer evolutionary hypotheses across a broad range of living organisms. Intraspecific sequence variation is assumed to be absent or negliable in most species, but few detailed studies have been conducted to assess the apportionment of ITS sequence variation within and between plant populations. *Buxus balearica* was chosen as a model species to assess the levels of infraspecific and intragenomic ITS variation in rare and endangered species occurring in disjunct populations around the Mediterranean basin. Intragenomic polymorphic sites were detected for western and eastern accessions of *B. balearica* and in two accessions of the sister species *B. sempervirens*. Overall, 19 different ribotypes were found in *B. balearica* after sequencing 48 clones, whereas 15 ribotypes were detected in 19 clones of *B. sempervirens*. The integrity and secondary structure stability of the ribosomal sequences suggest that they are not pseudogenes. The high number of ribotypes recovered through cloning suggested that some sequences could be chimeric or generated in vivo by partial homogenization through gene conversion or unequal crossing-over. Average sequence divergence among *B. balearica* clones was 0.768%, and the most divergent sequences differed by 1.62%. Available evidence does not suggest that *B. balearica* paralogues have been obtained from other extant *Buxus species* through interspecific hybridization. The presence of several ribosomal sequences in box implies that the molecular forces driving the concerted evolution of this multigene family are not fully operational in this genus. Phylogenetic analyses of cloned ITS sequences from *B. balearica* displayed very poor resolution and only two clades received moderate bootstrap support. Despite the marked intragenomic sequence divergence found, ribosomal data suggest a clear phylogeographic split in *B. balearica* between western and eastern accessions. The distinct, nonchimeric sequences that are postulated as being present in each biogeographic group suggest that box populations from Anatolia (eastern Mediterranean) are relict.

Key words: Intragenomic polymorphism — Ribosomal — Phylogeography — Concerted evolution

Introduction

The ribosomal region of the nuclear ribosomal cistron, composed by the 18S, 5.8S, and 25S genes, two internal spacers (ITS-1 and ITS-2), and the intergenic spacer (IGS), forms the unit that is further processed to produce the mature RNAs forming the cytoplasmic ribosomes of living organisms.Ribosomal loci are part of a multigene family formed by hundreds to thousands of tandem copies of ribosomal units; and usually several loci are present within plant genomes. Due to functional constraints, all ribosomal copies present within the genome are assumed to have

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identical sequences since the ribosomal multigene family usually evolve in concert (Arnheim 1983). This implies that all copies within and among ribosomal loci are expected to be homogenized throughout genomic mechanisms of turnover like gene conversion and unequal crossing-over (Dover 1994).

Sequences from the two internal transcribed spacers (ITS) have been widely used to infer phylogenetic and phylogeographic hypotheses across a broad range of living organisms, including fungi (Hughes et al. 2000; Peintner et al. 2001), algae (Coleman and Mai 1997), and green plants (Baldwin et al. 1995) Several factors have likely contributed to a spectacular rise in its use in plant evolutionary studies, including technical (availability of universal amplification primers, moderate size of both spacers) and operational (enough variation is usually detected at the species level suitable for phylogenetic analysis) reasons. Further, intraspecific variation is assumed to be absent or negliable in most organisms. As a consequence, species are usually represented by single or very few accessions when ITS sequences are analyzed in a phylogenetic context. However, few detailed studies have been conducted to assess the apportionment of ITS sequence variation within and between plant populations (e.g. Soltis and Kuzow 1993).

In addition, several authors have detected divergent intragenomic ITS copies in taxonomically distant groups (e.g., Buckler et al. 1997; Razafimandimbison et al. 2004). This intraindividual variation has been interpreted as the presence of multiple loci (paralogues), some of which have evolved without selective constraints and have accumulated mutations leading ultimately to nonfunctional loci (pseudogenes).

Cloning efforts are necessary to detect and characterize divergent ITS paralogues, but these are rarely conducted in routinary phylogenetic projects. Thus, levels of intraspecific and intragenomic ribosomal divergence could be underestimated if the ITS genotype is analyzed by direct sequencing on a non-representative sample of accessions.

The genus *Buxus* (Buxaceae) is widely represented on most continents but only two extant species are present in Europe and North Africa, *B. balearica* Lam. and *B. sempervirens* L., each of them having a nonoverlapping spatial distribution. The common box (*B. sempervirens*) is widely present throughout southern and western-central Europe, North Africa, and Western Asia, on dry and base rich (chalk and limestone) soils, usually in mesophyllous forests, mixed with deciduous species or forming pure populations. By contrast, the Balearic box (*B balearica*), occurs in very few disjunct populations around the Mediterranean basin (Fig. 1) including the southeast of the Iberian Peninsula, the Balearic Islands, Sardinia, North Africa (Riff, Middle Atlas, High Atlas, and Saharian Atlas), and southern Anatolia.

The underlying causes that explain these contrasting distributions in the two phylogenetically sister species (von Baltazhar et al. 2000) have not been fully appraised. However, climatic turnover, stochastic catastrophic events (e.g., fires), and anthropogenic activities could have shaped the distribution of *B. balearica* since Holocene times through the extinction of many populations linking the now disjunct western and eastern Mediterranean sites. However, alternative hypotheses should also be tested. Thus, on a theoretical basis successful long-range dispersal of propagules from Western sources could have been involved in the establishment of the Anatolian populations (see Mes et al. [1996] (for molecular evidence for plant dispersal from Macaronesia back to the eastern African continent). Finally, it could be argued that western and eastern populations of the Balearic box originated independently from separate ancestors; or from different gene pools of the same ancestor, located in separate areas; further reduction and fragmentation of western and eastern areas could have been influenced by climatic changes or human activity.

The facts that *B. balearica* has fragmented populations and a disjunct area make it a suitable model species to assess the levels of infraspecific and intragenomic variation using ITS sequences. Specifically, we sought (i) to assess what levels of ribosomal variation were present in a narrowly distributed Mediterranean species, (ii) to explore whether the pattern of ITS variation was related to geography, and (iii) to assess whether ITS markers could shed light on the origin of the disjunct distribution of the species.

Materials and Methods

Biological profile of Balearic box

Buxus balearica is a long-lived evergreen shrub or small tree that occurs on limestone from sea level to about 1200 m altitude (Benedí 1997). The inspection of the sampled populations showed an age structure with predominance of old adult plants. In fact, the studied populations of southeastern Spain and the Balearic Islands showed important problems of recruitment, mainly due to summer drought, which is an important constraint for seedling establishment (Lázaro et al., unpublished data). Although the species is ambophilous, being pollinated by both wind and insects, the former is usually the main pollen vector (Lázaro and Traveset 2005) and generates high quantities of pollen that can be dispersed to long distances. The species has the capacity of selfing and the proportion of selfed- seeds is not irrelevant (Lázaro and Traveset 2005). Fruits are dry and dehiscent; seed cases open suddenly, ejecting small black seeds up to a few meters away. In some populations ants can act as secondary dispersers, but the seeds do not attain long distances from the source plant (Lázaro et al., unpub-

Fig. 1. Distribution of *B. balearica* showing sampled populations. Location details and population codes are given in Table 1.

Table 1. List of investigated accessions of *Buxus balearica*, with locality, code, grid references, and altitude

Population	Code	Coordinates		Altitude (m)
North Africa				
Talcmbote (Morocco)	TA	5°16′26″W	35°16′06″N	160
Jebha (Morocco)	J	4°45′27″W	35°02′55″N	150
Boulemane (Morocco)	B	4°37′14″W	33°25′43″N	900
Cirque dc Jaffar (Morocco)	CJ	4°54′50″W	32°34′17″N	1100
Gorges du Todra (Morocco)	GT	5°35′09″W	31°35′51″N	1000
Iberian peninsula				
Frigiliana (Spain)	M	3°52′39″W	36°46′33″N	350
Ragol (Spain)	AL	2°41′30″W	36°59′03″N	440
Balearic islands				
Ternelles (Majorca)	TE	2°57′54″E	39°53′46″N	650
Artá (Majorca)	AR	3°21′01″E	39°46′43″N	340
Cap Ventós (Cabrera)	CV	2°57′54″E	39°09′43″N	50
Sardinia				
Carbonic (Italy)	CE	8°42′00″E	39°20′24″N	150
Monte Tassua (Italy)	MT	8°42′01″E	39°20′25″N	150
Anatolia				
Adrasan, Antalya (Turkey)	AD	30°30′55″E	36°19′45″N	300
Süphandere, Feke (Turkey)	FK	35°50′46″E	37°59′17″N	750
Antakya, Hat ay (Turkey)	AK	36°10′35″E	36°12′26″N	150

lished data). *Buxus balearica* also has the capacity to reproduce asexually by burying the lower branches and further rooting.

Plant material and DNA extraction

Representative specimens of *Buxus balearica* were sampled throughout their distribution range to assess ribosomal ITS variation (Table 1). Two accessions of the sister species *B. sempervirens*, from Spain (Montarejos) and Turkey (Maras), were used for comparative purposes. Total DNA was extracted from silica gel-dried leaves or herbarium specimens using the CTAB protocol of Doyle and Doyle (1987), scaled down to perform the process in 1.5-ml microfuge tubes.

Nuclear ribosomal ITS sequences

The region including ITS-1, 5.8S, and ITS-2 was amplified using the primer pair ITS1/ITS4 (White et al. 1990). In the cases where the DNA was of poor quality we amplified the ITS1 and ITS2 spacers separately using the primers ITS2 and ITS3 (White et al. 1990). PCR reactions were performed in 50 μl, containing 75 m*M* Tris-HCl (pH 9.0 at 25°C), 5 m*M* KCl, 20 m*M* $(NH_4)_2SO_4$, 0.0001% BSA, 1.5 m*M* $MgCl_2$, 5% DMSO, 200 μM of each dATP, dCTP, dGTP, and dTTP, 0,2 μM of each primer, approximately 50–100 ng of genomic DNA, and 0.75 units of *Taq* polymerase. Amplifications comprised an initial incubafion at 94°C for 3 min; then 40 cycles of 15 s at 94°C, 15 s at 56°C, and 30 s at 72°C. A final extension at 72°C for 5 min was included. The PCR products were separated on 1.0% agarose gels and purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). PCR products from a single individual from each population of the two box species were gel purified and ligated into the vector provided with the p-GEM-T (Promega) cloning kit. Plasmid DNA from individual recombinant colonies was isolated according to a miniprep protocol (High Pure Plasmid Isolation kit; Roche Diagnostics). For sequencing, purified PCR or cloned ITS products were reacted with BigDye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer, Applied Biosystems) using the amplification primers. The boundaries of the ITS sequences and ribosomal coding regions were determined by comparison to published sequences of *Buxus* (von Balthazar et al. 2000). DNA sequences have been deposited in GenBank under EF123164–EF1231197 codes.

Integrity and secondary structure stability of the ribosomal sequences

Sequences were compared for length variation and cytosine plus guanine content (C + G% hereafter). We explored the DNA sequences for the presence of several structural motifs. Thus, at the ITS1 region we searched for the presence and length of the conserved angiosperm motif (Liu and Schardl 1994) GGCRY-(4 to 7n)-GYGYCAAGGAA. This core sequence is most often associated with part of a hairpin structure, whereas the AAGGAA motif is predicted to stand as a non base-paired sequence in an internal

loop structure. It has been hypothesized that the whole or partial conserved motif serves as a critical processing signal in the enzymatic processing of the ribosomal RNA (Liu and Schardl 1994). Finally secondary structures of the ITS-2 RNA transcripts were examined for the presence of the tetranucleotide UGGU, which is preserved across the flowering plants. This motif is located within the C4 domain and is contained near the apex of helix III on the 5′ side (Mai and Colernan 1997). Predicted secondary structures of the ITS-1 and ITS-2 RNA transcripts and associated free energy values were evaluated using the minimum-free energy (MFE) algorithm (Zuker 1989) and the latest free energy rules (Mathews et al. 1999; Zuker et al. 1999). Fold predictions were made at the M. Zuker web server (Zuker 2003; http://www.bioinfo.rpi.edu/~zukerm/rna/) using the MFOLD program (version 3.1). Foldings were conducted at 37°C using a search to within 5% of the thermodynamic optimality set. Individual bases within foldings were annotated with the descriptor P-num (Jaeger et al. 1989, 1990) which indicates the propensity of individual nucleotides to participate in base pairs and, consequently, whether or not a predicted base pair is well determined. RNA structures were displayed with the RnaViz package (De Rijk and De Wachter 1997).

Phylogenetic data analysis

Buxus sequences were of similar length and were manually aligned and edited with DAMBE (Xia and Xie 2001). The network methods are designed for DNA haplotypes and are believed to work better with infraspecific phytogenies than standard parsimony algorithms. Thus, a parsimony network was implemented with the Network3x software (available at the web site http://www.fluxus-engineering.com) using the reduced median (RM) network method (Bandelt et al. 1995). Indels in the aligned sequences were coded as a fifth base irrespective of their length. Also, phylogenetic analyses using maximum parsimony were conducted using Fitch parsimony with equal weighting of all characters and of transitions/transversions as implemented in PAUP*4.0bl0 (Swofford 2002). Heuristic searches were replicated 100 times with random taxon-addition sequences, tree bisection-reconnection (TBR) branch swapping, and the options Multrees and Steepest Descent in effect. Support for monophyletic groups was assessed by bootstrapping (1000 resamplings of the data) using the heuristic search strategy as indicaied above (for discussion see Mort et al. 2000). In addition, phylogenetic reconstructions of ITS sequences were also performed using two independent approaches: distance-based (neighbor joining) and maximum likelihood (ML). For an ITS-sequence distance-based analysis, we used PAUP* 4.0bl0, the Kimura (1980) two-parameter distance model, and the neighbor-joining method (Saitou and Nei 1987). For ML, the model of nucleotide substitution that best fits the data (HKY + I + G) was selected with ModelTest (Posada and Krandall 1998). *B. sempervirens*, which according to von Balthazar et al. (2000) is sister to *B. balearica*, in both the ITS and the ndhF trees, was selected as the outgroup. The use of other more basal *Buxus* species available in GenBank was attempted, but sequence alignment was uncertain at several regions.

Results

Buxus balearica: Direct Sequencing and Cloning

Unreadable electrophoretograms were found in direct sequences at the ITS-l region in all the western Mediterranean accessions analyzed (Fig. 2a). Particularly, the sequences could not be read after a polyG tract located at the 5′ end. The displacement observed between peaks suggests that more than one ribosomal sequence, differing in length, could be present within the amplified ribosomal pool. However, eastern Mediterranean samples did not show any sequence displacement (Fig. 2b) and their ITS-1 sequences could be obtained with the forward sequencing primer. Also, all accessions from both western and eastern Mediterranean samples showed double peaks in. different sites at the ITS-1 and ITS-2 regions. Overall, we found 11 intragenomic polymorphic sites for western populations (5 in ITS1 and 6 in ITS2), whereas only 3 were found in eastern populations, 2 in ITS-1 and in 1 ITS-2 (Table 2). Given such sequence ambiguities it was necessary to clone the PCR products in order to assess the number of intragenomic ribotypes present.

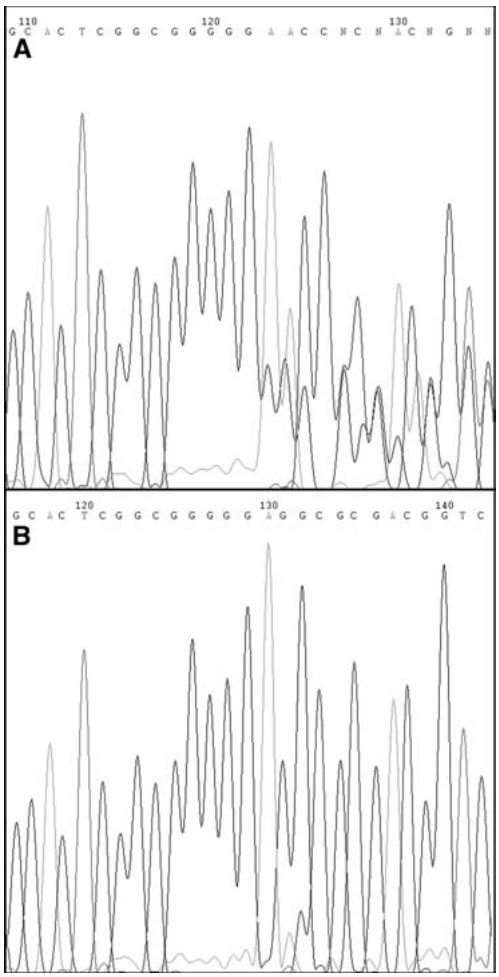

Fig. 2. Electrophoretograms from direct sequences of *B. balearica* at the indel region of the ITS-1 spacer. **A** sequences from western accessions showing base displacement due to the presence of ITS paralogues of different length. **B** eastern accessions.

We cloned the ribosomal products amplified from six individuals coming from separate geographic regions: one from Morocco (Cirque Jaffar; CJ), one from the Iberian Peninsula (Málaga; M), one from the Balearic Islands (Ternelles; TE), one from

Table 2. Polymorphic sites in direct ribosomal ITS sequences from *B. balearica* accessions

	ITS-1						ITS-2					
	47	48	111	114	141	238	276	369	377	456	463	486
Western Mediterranean												
Talembote	T	G	G/–	C	A	G	K	C	S	Y	C	M
Jebha	K	G	G/–	C	R	S	K	C	S	Y	C	M
Boulemane	K	S	G/–	C	A	S	K	C	S	Y	C	M
Cirque de Jaffar	T	G	G/–	C	A	S	K	C	S	Y	C	M
Gorges du Todra	T	G	G/–	C	A	S	K	C	S	Y	C	M
Frigiliana	K	S	G/–	C	R	S	K	C	S	Y	C	M
Rágol	T	G	G/–	C	R	S	K	C	S	Y	C	M
Ternelles	T	G	G/–	C	R	S	K	C	S	Y	C	M
Artá	T	G	G/–	C	R	S	G	C	S	Y	Y	M
Cap Ventós	T	G	G/–	C	R	S	K	C	S	Y	C	M
Carbonia	T	G	G/–	C	R	S	G	C	S	Y	Y	M
Monte Tassua	T	G	G/–	C	R	S	G	C	S	Y	Y	M
Eastern Mediterranean												
Adrasan	T	G	—	S	R	C	G	M	C	T	C	C
Süphandere	T	G	—	S	R	C	G	M	C	T	C	C
Antakya	T	G	—	G	A	C	G	A	C	T	C	C

Note. Sequence ambiguities follow IUPAC codes. R denoted A or G, Y denotes C or T, K denotes G or T, M denotes A or C, and S denotes G or C. Numbers refer to the aligned sequences of *B. sempervirens* and *B. balearica* (without the 5.8S region).

Table 3. Ribotypes found in 48 cloned sequences of *B. balearica* accessions: numbers refer to the aligned sequences of *B. sempervirens* and *B.balearica* (without the 5.8S region)

	ITS-1						ITS-2					
Ribotype	47	48	111	114	141	238	276	369	377	456	463	486
1	T	G	—	C	G	C	G	C	C	T	C	C
2	T	G	—	C	G	G	G	C	C	T	C	C
3	T	G	—	C	G	C	G	C	C	C	C	C
4	T	G	—	C	A	C	G	C	C	T	C	C
5	T	G	—	G	A	C	G	C	C	T	C	C
6	T	G	—	G	A	C	G	A	C	T	C	C
7	T	G	G	C	A	C	G	C	C	C	C	C
8	T	G	G	C	A	G	G	C	C	C	C	A
9	T	G	G	C	A	C	G	C	C	C	C	A
10	T	G	G	C	A	C	G	C	C	T	T	A
11	T	G	G	C	A	C	G	C	C	C	T	A
12	T	G	—	C	G	C	G	C	C	C	T	A
13	T	G	—	C	A	G	G	C	C	T	C	C
14	T	G	—	C	A	G	G	C	G	T	C	C
15	T	G	—	C	A	G	G	C	G	C	C	C
16	G	C	—	C	A	G	G	C	G	T	C	C
17	G	C	—	C	A	C	T	C	G	T	C	C
18	G	C	—	C	A	G	T	C	G	C	C	C
19	G	C	—	C	A	C	T	C	G	C	C	C

Sardinia (Carbonia; CE) and two from two Turkish populations (Feke, FK; and Antokya, AK), The number of analyzed clones per individual ranged from 3 to 11. Altogether, we sequenced 48 clones yielding 19 different ribotypes (Table 3). Two of the clones (M14 clon8, AD3_2 clon7) were identical to the *B. balearica* sequence (GenBank AF245423) obtained by Von Balthazar et al. (2000). The number of ribotypes found within western Mediterranean individuals ranged from four (CJ) to seven (from TE), with an average of 5.5. By contrast, fewer ribotypes per individual (range, 1–4; average, 2.5) were found in the eastern accessions. The length of ITS-1 varied from 262–263 bp, the 5.8S was 160 bp, whereas the ITS-2 was uniformly 239 bp. The number of variable sites was 12, 6 in each of the ribosomal spacers. No variation was found in the 5.8S gene, and accordingly, this region was henceforth excluded in the

phylogenetic analysis. The G + C% was similar in all clones and ranged from 66.0% to 66.8%. Both ITS length and G + C values were within the known ranges for flowering plants, although *Buxus balearica* data approach the higher reported values (Baldwin et al. 1995). Across the whole data set, we retrieved between one (Feke) and seven (Ternelles) different intragenomic ITS sequences, and the average number per individual was four. Average sequence divergence among clones (Kimura two-parameter model) was 0.768%, and the most divergent sequences differed by 1.62%. Interestingly, this maximum sequence divergence was found (i) from clones belonging to the same individual (CE), (ii) from clones belonging to different individuals from the western Mediterranean (TE versus M, CE, CJ), and (iii) from clones retrieved from individuals from western and eastern accessions (AD versus CE, FK versus CE).

Buxus balearica: Comparison of Direct vs Cloned Sequences

Polymorphic sites and ambiguous readings in the direct sequences were resolved when cloning procedures were applied to ribosomal PCR products. At each of the polymorphic sites, the different bases found in the cloned sequences mostly matched the diversity found in the electrophoretograms of the direct sequences (Table 5). However, in some accessions cloning revealed intragenomic polymorphis that were not apparent in the direct sequences. The fact that two types of cloned sequences differing in length (1 bp) were found in western *Buxus* accessions agrees with predictions suggesting that the displacement observed between peaks at the ITS-1 region were due to the presence of sequences of unequal length. A close inspection of both direct and cloned sequences revealed conspicuous differences between western and eastern boj accessions: (i) western accessions showed more ribotypes (17) than eastern samples (4); (ii) the longest ribosomal paralogues were restricted to the Western area; (iii) overall, ITS sequences from Western and Eastern accessions differed at 12 sites (Table 3), all of which showed intragenomic polymorphisms in western or eastern samples and (iv) most of the ITS ribotypes were exclusive to either western or eastern samples (Table 4). Thus, ribotypes 2, 3, and 7–19 were restricted to western samples, whereas ribotypes 5 and 6 were exclusively eastern ones. By contrast, ribotypes 1 and 4 were present in both areas. Only one (AD3_2 clon3) of the 11 cloned products from eastern samples belongs to ribotype 1, mainly present in the western area. Similarly, only one (M14 clon8) of the 37 sequenced clones from western samples showed ribotype 4, also present in a single eastern clone (AD3_2 clon7). Recombinant sequences may be deconstructed in parental sequences if a large number of clones is examined and if direct sequences are available for comparison. Five ribotypes (1, 2, 6, 11, and 18) that were not chimeric could generate the electrophoretograms displayed by direct sequencing. Thus, direct ITS sequences of western accessions could be interpreted by the sum of ribotypes 2, 11, and 18 (Table 5). Likewise, a minimum number of two ribotypes (1 and 6) was required to explain the electrophoretic pattern of eastern samples (Table 5).

Cloned Sequences of B. sempervirens

The ITS region from western Mediterranean (Iberian peninsula) and a eastern Mediterranean (Turkey) accessions from the related *B. sempervirens* was cloned and sequenced. Fifteen different ribotypes were recovered from 19 analyzed clones (Table 6). No single clone was identical to the *B. sempervirens* sequence (GenBank AF245429) reported by Von Balthazar et al. (2000). Sequence diversity was greater in the western Mediterranean (10 ribotypes of 10 clones) than in the eastern Mediterranean accession (4 ribotypes of 9 clones). The number of polymorphic sites was 29, of which 26 were point mutations and the other involved indels of 6 and 2 bp. Average sequence divergence among all clones (Kimura, two-parameter model) was 0.965%. Intragenomic sequence divergence within the Iberian sample ranged from 0.2% to 2.857% and was greater than that obtained within the Turkish sample (0.01% to 0.611%). Sequence divergence between western and eastern clones ranged from 0.0% to 2.273%.

Structural Integrity and RNA Secondary Structure of the Ribosomal Sequences

Retrieved sequences from *B. balearica* and *B. sempervirens* showed several structural landmarks typical of angiosperm ribosomal ITS spacers (Mayol and Rosselló 2001). First, we noted the presence in the ITS-1 region of the universal motif of Liu and Schardl (1994), GGCRY-(5n)-GYGYCAAGGAA [GGCGCGATCGGCGTCAAGGAA]. Its secondary structure in optimal or suboptimal foldings (Fig. 3) agrees with predictions: 12 bases are associated with part of a hairpin structure, whereas the remaining GTCAAGGAA motif is predicted to stand as a non-base-paired sequence in an internal loop structure. *B. sempervirens* clones with a 6-bp deletion showed a different secondary structure of the Liu and Schardl (1994) motif: all bases were predicted to show an association with a stem structure. Second, the 5.8S coding region showed the presence of the conserved EcoRV site (GATAC). Third, the univer-

Table 4. Distribution of ITS ribotypes in six *B. balearica* accessions coming from separate geographic regions: Morocco (CJ), the Iberian peninsula (M), Balearic Isalnds (TE), Sadinia (Carbonia, CE), and Turkey (AD, FK)

	Ribotype																		
Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Western Mediterranean																			
CJ									X							X	X		X
M	X			X										X	X	X		X	
TE	X	X	X								X	X	X						X
CE	X		X				X	X		X									
Eastern Mediterranean																			
AD	X			X	X	X													
FK						X													

Table 5. Comparison of direct (D) and cloned sequences (ribotypes: R) in six *B. balearica* accessions

	ITS-1						ITS-2					
	47	48	111	114	141	238	276	369	377	456	463	486
Western Mediterranean												
Cirque de Jaffar												
D	T	G	G/–	C	A	S	K	C	S	Y	C	M
R-9	T	G	G	C	A	C	G	C	C	C	C	A
R-16	G	C	—	C	A	G	G	C	G	T	C	C
R-17	G	C	—	C	A	C	T	C	G	T	C	C
R-19	G	C	—	C	A	C	T	C	G	C	C	C
Frigiliana												
D	K	S	G/–	C	R	S	K	C	S	Y	C	M
R-1	T	G	—	C	G	C	G	C	C	T	C	C
R-4	T	G	—	C	A	C	G	C	C	T	C	C
R-14	T	G	—	C	A	G	G	C	G	T	C	C
R-15	T	G	—	C	A	G	G	C	G	C	C	C
R-16	G	C	—	C	A	G	G	C	G	T	C	C
R-18	G	C	—	C	A	G	T	C	G	C	C	C
Ternelles												
D	T	G	G/–	C	R	S	K	C	S	Y	C	M
R-1	T	G	—	C	G	C	G	C	C	T	C	C
R-2	T	G	—	C	G	G	G	C	C	T	C	C
R-3	T	G	—	C	G	C	G	C	C	C	C	C
R-11	T	G	G	C	A	C	G	C	C	C	T	A
R-12	T	G	—	C	G	C	G	C	C	C	T	A
R-13	T	G	—	C	A	G	G	C	C	T	C	C
R-19	G	C	—	C	A	C	T	C	G	C	C	C
Carbonia												
D	T	G	G/–	C	R	S	G	C	S	Y	Y	M
R-1	T	G	—	C	G	C	G	C	C	T	C	C
R-3	T	G	—	C	G	C	G	C	C	C	C	C
R-7	T	G	G	C	A	C	G	C	C	C	C	C
R-8	T	G	G	C	A	G	G	C	C	C	C	A
R-10	T	G	G	C	A	C	G	C	C	T	T	A
Eastern Mediterranean												
Adrasan												
D	T	G	—	S	R	C	G	M	C	T	C	C
R-1	T	G	—	C	G	C	G	C	C	T	C	C
R-4	T	G	—	C	A	C	G	C	C	T	C	C
R-5	T	G	—	G	A	C	G	C	C	T	C	C
R-6	T	G	—	G	A	C	G	A	C	T	C	C
Süphandere												
D	T	G	—	S	R	C	G	M	C	T	C	C
R-6	T	G	—	G	A	C	G	A	C	T	C	C

Note. Sequence ambiguities follow IUB codes. R denotes A or G, Y denotes C or T, K denotes G or T, M denotes A or C, and S denotes G or C.

Table 6. Ribotypes found in 19 cloned sequences of *B. sempervirens* accessions: numbers refer to the aligned sequences of *B. sempervirens* and *B. balearica* (without the 5.8S region)

Accession	Ribotype	5	3 2	3 8	7 7	9 9	12 12	12 13	12 14	12 15	12 16	12 17	12 18	16 5	18 6	18 9	19 1	19 2	21 8	22 0	23 9	23 1	24 8	24 5	25 5	25 7	26 4	26 4	27 3	27 6	27 9	30 7	34 3	34 4	34 6	45 1	55 3	55 1	57 0	59 8	64 3	64 4
Turkey, Maros	20	A	G	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	G	A	T	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	21	A	G	T	T	T	—	—	—	—	—	—	—	G	A	C	—	—	G	A	T	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	22	A	G	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	A	T	T	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	23	A	G	T	T	T	—	—	—	—	—	—	—	G	A	C	—	—	G	A	T	C	A	G	G	T	A	A	A	A	C	C	T	A	T	G	T	C
	24	G	G	T	T	T	—	—	—	—	—	—	—	G	A	C	—	—	G	A	T	C	A	G	A	C	A	G	A	A	C	C	T	A	T	G	T	C
Spain, Montarejos	25	A	G	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	A	A	C	G	A	T	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	26	A	A	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	A	T	C	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	27	A	A	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	A	T	C	C	G	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	28	A	G	T	T	T	T	C	G	C	G	T	C	G	A	T	T	A	G	A	T	C	A	G	A	T	A	A	A	G	T	T	T	A	C	A	—	—
	29	A	G	T	C	T	T	C	G	C	G	T	C	C	A	T	—	—	A	T	T	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	30	A	G	T	T	T	T	C	G	C	G	T	C	G	T	T	T	A	G	A	T	C	A	G	A	T	G	A	A	G	T	T	T	G	C	A	T	C
	31	A	G	A	T	G	T	C	G	C	G	T	C	G	A	T	—	—	G	A	T	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	C
	32	A	G	A	T	G	T	C	G	C	G	T	C	G	A	T	—	—	A	T	C	C	A	G	A	T	A	A	A	A	C	C	T	A	T	G	T	T
	33	A	G	T	T	T	T	C	G	C	G	T	C	G	A	C	—	—	G	A	T	C	A	G	A	T	A	A	G	A	C	C	T	A	T	G	—	—
	34	A	G	T	T	T	T	C	G	C	G	T	C	G	A	T	—	—	G	A	T	C	G	G	A	T	A	A	A	A	C	C	C	A	T	G	—	—

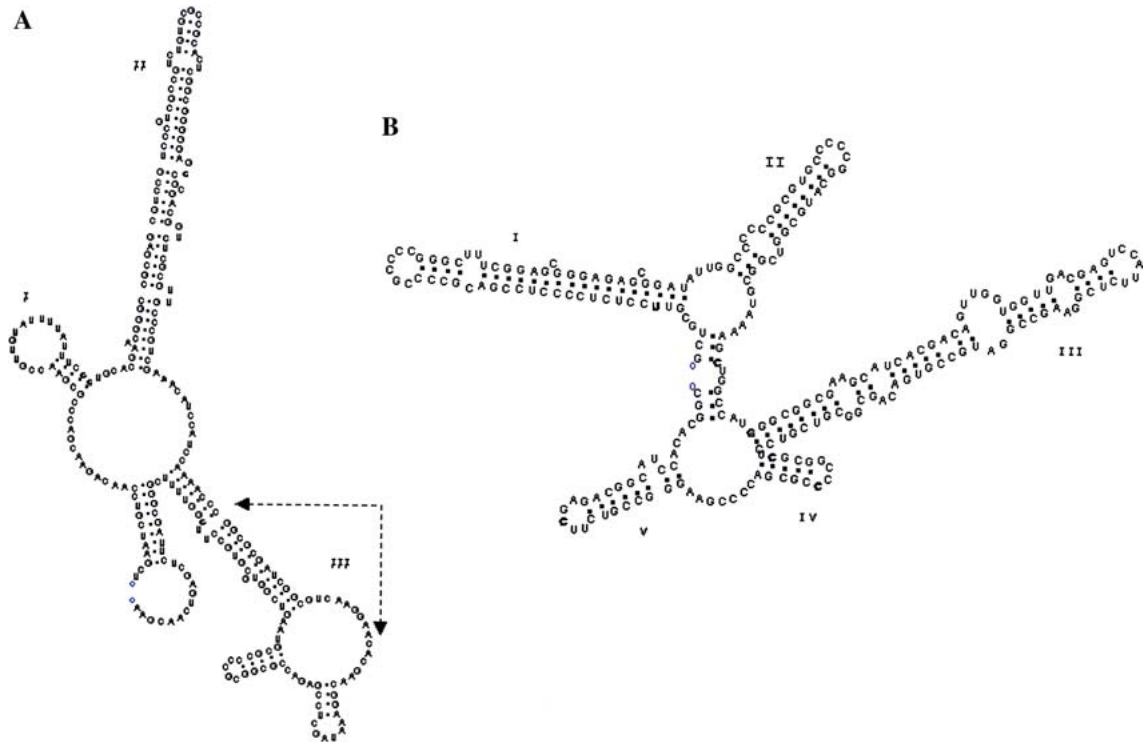

Fig. 3. Putative secondary structures of the ITS RNA transcripts of *B. balearica*. Polymorphic sites are in bold. **A** ITS-1. The conserved motif of Liu and Schardl (1994) [GGCRY-(4 to 7n)-GYGYCAAGGAA] is included between dashed arrows. **B** ITS-2.

sally conserved pyrimidine-pyrimidine mismatch (in *Buxus double* pairing) in helix II of the predicted structure of the ITS-2 RNA transcript (C U, C C) was detected in all sequences. Fourth, the predicted secondary models of the ITS-2 RNA transcripts conform well with a cruciform (four to five helix) structure present in most algal and plant vascular species (Fig. 3). In addition, helix III is the longest stem-loop, as early reported for flowering plants. Fifth, thermodynamically, the secondary structures of the RNA transcripts of all sequences were similar and stable, as inferred from the free energy values (> 100 kcal/mol) of the folded sequences. Finally, we noted the presence of the tetranucleotide UGGU,

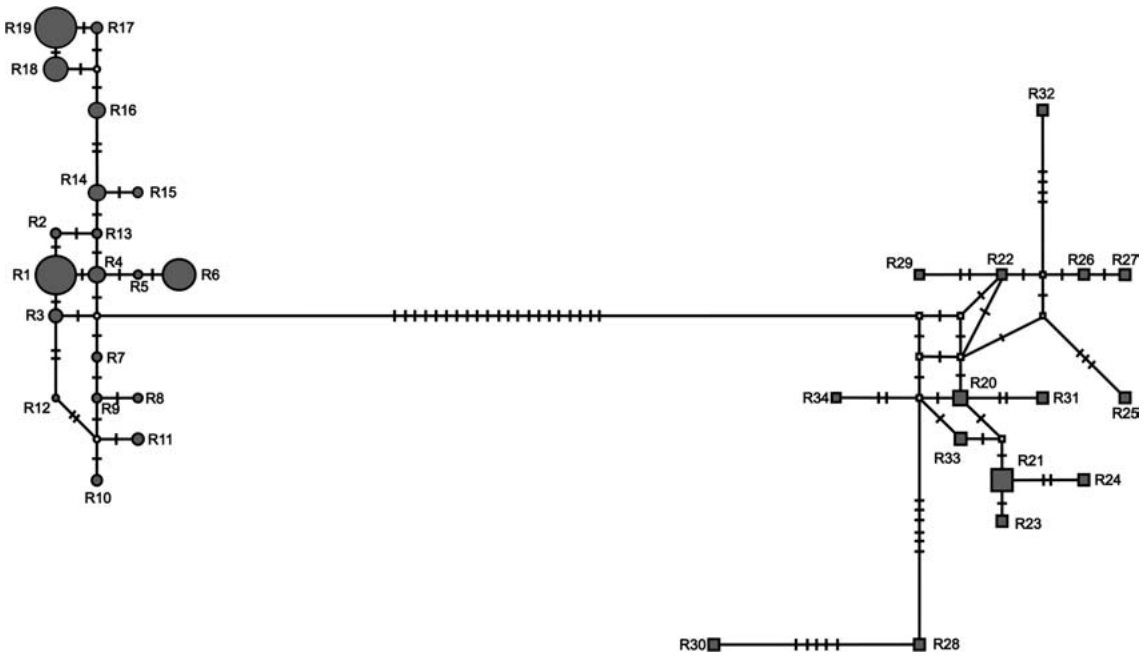

Fig. 4. Median parsimony network depicting ribotype relationships found in *B. balearica* (circles) and *B. sempervirens* (squares) accessions. Open circles and squares indicate inferred but not sampled haplotypes. Each line denotes a mutational event among connected ribotypes.

contained near the apex of helix III of the ITS-2 RNA transcript on the 5′ side. Only a single divergent structural motif in *B. balearica* and *B. sempervirens* sequences was detected. The conserved motif in the 5.8S region GAATTGCAGAATTC was replaced in all sequences (either cloned or from direct readings) by the highly similar GAATTGCAGAA_TCC. Inspection of the ribosomal sequences in GenBank (from Von Balthazar et al. 2000) revealed that most *Buxus* species had the latter motif, but no species showed the conserved motif. In addition, deviant sequences from these two patterns at four sites were found in four Species: *B. henryi* (AF245409), *B. perrieri* (AF245432), *B. (Nothobuxus) acuminata* (AF245434), and *B. hildebrandtii* (AF245415). Compensatory mutations were not inferred according to the predicted RNA structures.

Phylogenetic Analysis of Buxus Ribotypes

The parsimony network of *Buxus* ribotypes is depicted in Fig. 4. Clones from each species were grouped in two distinct groups that differ by a minimum of 25 mutational events. Complex patterns were present in the network, including unresolved loops that are the result of several homoplasious sites. Inferred, but not sampled haplotypes were more common in the *B. sempervirens* (eighth) than in the *B. balearica* (three) network. In *B. balearica*, ribotypes restricted to the eastern Mediterranean (R4, R5, R6) showed a noninternal group that was closely located near the inferred ribotype linking the *B. sempervirens* network. In *B. sempervirens*, no discrete groupings of the western and eastern Mediterranean ribotypes could be seen. The joint analysis of both *B. sempervirens and B. balearica* ribotypes using other phylogenetic methods always showed that sequences from each species formed well supported monophyletic clades. However, they do not improve relationships between haplotypes and showed a factual polytomy for most branches since branch lengths were short in most clades and were not statistically supported. Cladistic (MP) analysis of cloned *B. balearica* ITS sequences yielded 204 trees (CI = 0.53 and RI = 0.46, including uninformative characters). The strict consensus tree (not shown) displayed very poor resolution, mainly due to conflict, and only two clades received moderate bootstrap support (69% and 62%). One of the clades comprised ribotypes 16, 17, 18, and 19 (western Mediterranean), whereas the other included two ribotypes (5 and 6) restricted to the eastern Mediterranean accessions. These clades were also recovered in the neighbor-joining tree (Fig. 5), but with a slightly higher bootstrap support (74%), and in the ML tree ($-\ln L$ 899.63542). In all these analyses western and eastern ribotypes were not monophyletic and did not show a sister relationship. In fact, the latter were embedded within a clade including the former.

Fig. 5. Neighbor-joining tree derived from Kimura (1980) two-parameter distances of cloned ITS ribotypes from *B. balearica* (squares) and *B. sempervirens* (diamonds). Filled symbols indicate ribotypes present in Turkish populations. Values above the nodes indicate bootstrap values higher than 70% obtained after 1000 replicates.

Discussion

The Nature of Ribosomal paralogous Sequences in Buxus

The ITS region forms part of the 455 cistron, with thousands of tandem copies clustering in arrays that, in turn, form the basis of the ribosomal loci. By means of molecular cytogenetic methods (like FISH), it has been ascertained that usually several ribosomal loci, either transcriptionally active (NOR region) or inactive, are present within plant genomes (e.g., Zhang and Sang 1999; Raina et al. 2001; Benabdelmouna et al. 2001; Singh et al. 2001; Nakamura et al. 2001). This feature, however, seldom poses serious problems in practical phylogenetic reconstruction. In fact, and in the absence of cloning, a single consensus ITS sequence is usually retrieved from PCR products from single individuals (but see Buckler et al. 1996, 1997; Mayol and Rosselló 2001), which is then used as raw data in phylogenetic reconstruction, This is due to the fact that sequences from a multigene family usually evolve in concert (Arnheim 1983) and all copies within and among ribosomal loci are homogenized throughout genomic mechanisms of turnover, like gene conversion and unequal crossing-over (Dover 1994). This is why one should expect to find within-species homogeneity and between-species diversity for multigene sequences. Sometimes, this molecular scenario could break down if (i) the rate of mutation among copies is faster than that driving the concerted evolution of the array, (ii) duplicate ribosomal loci evolve without selective constraints and accumulate mutations leading ultimately to pseudogenes, and (iii) homeologous (xenologous) loci from other species are incorporated into the nuclear genome through hybridization-mediated processes like introgression, amphidiploidy, and allopolyploidy. In such cases, the molecular homogenizing mechanisms could be relaxed and two or more ribotypes can be present within organisms (e.g., Sang et al. 1995; O'Kane et al. 1996; Campbell et al. 1997; Szalanski et al. 2001).

In their phylogenetic study of *Buxus*, Von Balthazar et al, (2000) noted that the amplification of ITS from some taxa (not explicitly specified) yielded products of a heterogeneous composition, indicating the presence of more than one DNA template. Although the authors claimed that these heterogeneous products were cloned and at least two clones were sequenced, no sound evidence of ribosomal paralogy was presented and discussed since each taxon of *Buxus* used was represented by a single GenBank accession and a single ITS sequence in the phylogenetic tree depicted. From these facts we infer that either (i) all sequenced clones from a single accession shared the same ribotype or (ii) the authors retained the most frequent sequenced ribotype as being typical for each accession and discarded the other ITS variants.

In this study, however, contrary to expectations, we have found that significant intragenomic variation in ribosomal sequences is present in both a closely restricted and a widely distributed shrub. The available evidence strongly suggests that the intragenomic ITS variability found is due not simply to PCR or sequencing artifacts, but to the presence of divergent ribosomal copies. However, the high number of ribotypes recovered through cloning suggested that some sequences could be chimeric (in vitro PCR recombination) or generated in vivo by partial

homogenization through gene conversion or unequal crossing-over.

Several lines of evidence concur that all *Buxus* ribotypes recovered in this work are functional among angiosperm ITS sequences. The length and G + C content of the spacers and the coding 5.8S region, the presence of virtually all structural landmarks in the primary DNA sequence, the null rate of substitutions and deletions in the 5.8S region and in the highly conserved motifs, the high thermodynamic stability, and the conserved cruciform foldings of the secondary RNA structures do not suggest that they are ribosomal pseudogenes.

Moreover, an inspection of the sequences of the 5.8S coding region of the *Buxus* taxa reported by Von Balthazar et al. (2000) revealed some surprising results concerning this conserved gene. First, the aligned 5.8S sequences showed 47 variable sites, resulting in eight distinct ribotypes from only 13 taxa. This divergence is of the same order as that found in a comparison of 5.8S sequences in nine diverse angiosperms across a wide systematic range, from monocots to dicots (Hershkovitz and Lewis 1996). It is also illustrative to compare the *Buxus* value with that obtained for a sample of an Asteraceae data set, showing 8 of 167 variable sites (Baldwin et al. 1995). Second, some sequence (*B. hildebrantii*) showed indels of 2 bp, which were associated to a very divergent 5.8S ribotype. Third, as reported above, deviant sequences from the angiosperm conserved motif GAATTGCAGAATTC were found in all *Buxus* species, where *B. henryi, B. perrieri, B. (Nothobuxus) acuminata*, and *B. hildebrandtii* are the most divergent. According to Hershkovitz et al. (1999) all these unusual features of the 5.8S gene suggest that some of the rDNA copies retrieved by Von Balthazar et al. (2000) might not be functional and are not likely to be expressed. However, in the absence of experimental work dealing with RNA expression (e.g., those carried out by Hartmann et al. [2001] and Muir et al. [2001]), there is no sound evidence of the functionality of the recovered paralogous loci in *Buxus* in general and in *B. balearica* and its sister species in particular.

Ribosomal Gene Families and Concerted Evolution im Buxus

There is no reason to suggest that the within-individual polymorphism detected would be restricted within the genus to *B. balearica* and *B. sempervirens*. Available evidence suggests, but does not prove, the likely presence of several gene families that are associated with different rDNA loci in the *Buxus* genome, but it could not be formally discarded that the gene families are located within the same array. In fact, there is not a clear relationship between the number of ribosomal families (including the 55 gene) and the number of rDNA loci (e.g., Hanson et al. 1996; Lim et al. 2000; Matyasek et al. 2002). The assumption that the observed ribosomal variation in *B. balearica* is compatible with the presence of at least three (western accessions) or two (eastern accessions) rDNA loci in the nuclear genome should be substantiated with other more appropiate molecular techniques (FISH, restriction mapping, Southern blotting) to quantify the location and number of the rDNA operons.

Due to the presence of lower chromosome numbers in Buxaceae (e.g., *Sarcococca;* $2n = 14$), cytogenetic data suggest that, like other species of box so far known, *B. balearica* is polyploid (tetraploid, $2n = 28$ [Darlington and Wylie 1955; Huang et al. 1988; Dempsey et al. 1994]). Thus, our results concerning the within-individual ribosomal variation are consistent with the polyploid nature of the *Buxus* genome, A detailed inspection of available ribosomal sequences from box species does not Show any evidence that *B. balearica* paralogues have been obtained from other extant species through interspecific hybridization. In fact, and although the sampling in *B. sempervirens* has not been extensive, the parsimony network recovered (Fig. 2) suggests a clear ribotype differentiation between the two species without apparent signs of nuclear capture. Increase in NOR sites could be due not only to polyploidy, since the increase in the number of rDNA loci could also have resulted either from duplication of the major NOR arrays, from the amplification of minor loci consisting of a few rDNA copies, or from the translation of rDNA repeats from chromosomes bearing rDNA loci to chromosomes lacking them (Dubcovsky and Dvorak 1995; Hanson et al. 1996).

From the data available, we can speculate that the amplification of ribosomal loci was present in the ancestors of *B. balearica* and *B. sempervirens* and the molecular divergence between loci was subsequent to the most recent speciation (shallow paralogy; *sensu* Bailey et al. 2003).

Independently of their origin, the presence of several ribosomal sequences in box implies that the molecular forces driving the concerted evolution of this multigene family are not fully operational, at least in these two species. If the non-transcriptionally active NOR loci are selective neutral, they would result in rDNA heterogeneity and would speed up divergence, thereby becoming pseudogenes, as the inspection of some sequences from Von Balthazar et al. (2000) suggests.

In artificial plant hybrids or recent established polyploids, there are reports indicating that concerted evolution between ribosomal gene families toward one of the parental sequences could occur in very few generations. In fact, homogenization of ITS poly-

morphisms in *Armeria* is already observed in the F_2 generation (Fuertes et al. 1999). The reasons why, in *B. balearica* and *B. sempervirens* (and presumably in all the genus), the ribosomal spacers have escaped from homogenization are open to further research. It is likely that the 45S ribosomal families are on different loci located in separate chromosomes, preventing concerted evolution, since it has been pointed out that the homogenization of the rDNA repeats through gene conversion and unequal crossing-over occurs more effectively within than between loci (Otha and Dover 1983). In addition, the chromosomal location of rDNA loci could also be involved since it has been suggested that it has a more substantial impact than the number of loci on the tempo of concerted evolution through either unequal crossing-over or gene conversion (Zhang and Sang 1999). Since *B. balearica* and *B. sempervirens* have a relatively long generation time, this could imply a detectable generation-time effect that could slow down rates of concerted evolution (Sang et al. 1995).

The Phylogeogrphic Split in Buxus balearica

The phylogenetic analyses of the cloned ribosomal paralogues does not show a sister relationship between accessions from each side of the Mediterranean basin; Rather, the eastern clones were nested within those from western accessions, and a strict monophylly of each group of paralogues was not recovered. Network analysis, which has been postulated to describe relationships between sequences of multigene families in a more realistic fashion than conventional tree building (Allaby and Brown 2001), also show congruent results. Our results in *B. balearica* and *B. sempervirens* contrast with those found in *Larix*, where the phylogenetic analysis of the ribosomal paralogues showed that clones from all species were mixed together and no species relationships were resolved despite the fact that some clades had a high bootstrap value (Wei and Wang 2004). Given the potentially high number of ribosomal loci found in *B. balearica*, violation of the assumed orthology of the recovered clones is a serious concern and would have serious consequences for reconstructing their relationships using conventional phylogenetic analysis, as aptly advised by Dubcovsky and Dvorak (1995). In addition, lack of strong phylogenetic resolution within *B. balearica* could be related to PCR artifacts. In vitro-mediated recombination is well known to occur when nonidentical templates from multigene families are amplified by PCR, causing potential problems in phylogenetic recontruction (Cronn et al. 2002). In addition, a clear distinction is not always possible between chimeric sequences and partial homogenizing sequences through concerted evolution. In either case, the use of such intermediate sequences introduces noise when a phylogenetic analysis is required.

However, and despite the marked intragenomic sequence divergence found, ribosomal data suggest a clear phylogeographic split in *Buxus balearico* between western and eastern samples. The distinct, nonchimeric sequences that are postulated to be present in each biogeographic group allow an interpretation of the disjunct areas occupied by *B. balearica*. We hypothesize that the Turkish populations of Balearic box are not the result of a recent (Pleistocene) long-range dispersal of propagules from western sources. This inference agrees with the poor seed dispersal capacity that characterizes *B. balearica* populations, which show a disperal distance from the shrub source of only a few meters (Lázaro et al., unpublished data). Given the highly appreciated use of this wood by humans for several purposes, a likely anthropogenic introduction of the Turkish populations could be envisaged from a western provenance in historical times. However, this hypothesis is clearly rejected in the light of the ribosomal data.

Available evidence from the nuclear genome suggests that box populations from Anatolia are relict and are remnants of a formerly widespread species that became extinct throughout most of the area. The position of the ribotypes in the network analysis suggests that western and eastern populations of the Balearic box did not originate independently from separate ancestors. Rather, the suggested scenario is that they diverged from a common gene pool after either a major biogeographic break or a dramatic bottleneck that led to sudden, stochastic fluctuations in an evolutionary lineage. The network analysis has shown that the eastern ribotypes from *B. balearica* (i) are not located in the terminal tips of the network and (ii) lie close to the node connecting the *B. balearica–B. sempervirens* split. Both pieces of evidence suggest that the eastern ribosomal paralogues are relictual and are likely to have retained features of the ancestral ribosomal gene pool of the species.

Preliminary data from the cpDNA genome (trnL intron, trnL-trnF spacer; unpublished data) is congruent with the western/eastern phylogeographic split and support the view that data from the paralogous ribosomal loci are not artifactual and originated from a preferential PCR amplification of gene families or cloning of several types of sequences

Buxus balearica, in contrast to its sister species *B. sempervirens*, is not a cold-tolerant taxon, whose populations are restricted to patches of mesic and sclerophyllous vegetation. In fact, its current distribution does not extend beyond 39°N of latitude, suggesting that past or present climatic events have shaped its distribution. Quaternary glaciations have heavily influenced the distribution of a large component of the

European biota (Hewitt 1999, 2000), and this has been interred for a large number of tree and shrub species (Taberlet et al. 1998). Extant populations of *B. balearica* are exclusively located on unglaciated southern refugia that were not severely affected by the Quaternary cold periods. Its presence in the western Mediterranean basically agrees with the postulated southern European refugia (Taberlet et al. 1998). Moreover, its relictual occurrence in the Anatolian peninsula is congruent with a main eastern refugia located in the geographically related Balkanic peninsula (Taberlet et al. 1998). Interestingly, some other plant vascular taxa that are considered to be putative tertiary relicts mirror the disjunct distribution of *B. balearica*, Thus, *Rhododendron ponticum* L. occurs naturally in three unglaciated southern refugia in Europe around the Black Sea, the Balkans, and the Iberian peninsula *Viola* section *Delphiniopsis* W. Becker occurs in the Iberian peninsula (*V. cazorlensis* Gand.) and in the Balkans (*V. kosanninii* and *V. delphinantha)*.

The Anatolian populations of Balearic box were once distinguished as a separate species (*B. longifolia* Boiss.) from *B. balearica* on the basis of several foliar features. However, the range of variation found in the western Mediterranean plants did not justify the separation of the Turkish populations from the latter and were retained within *B. balearic* without formal recognition (Davis 1982). Palynological data agree with this view (Brüchner 1993). Given the levels of ribosomal diversity found in *B. balearica* and *B. sempervirens*, the molecular data do not support (but do not strictly refute) the distinction of *B. longifolia* as a separate taxa from *B. balearica*. Perhaps other molecular markers of the nuclear genome not belonging to multigene families would more clearly substantiate whether morphological data parallel molecular discontinuities.

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