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Biogeographic divergences in the Iberian flora. A morpho-anatomic, ISSR-based, and environmental study of Iberian *Buxus sempervirens* L.

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Abstract: Iberian *Buxus* spp. are represented by *B. sempervirens*, restricted to the Cantabrian, eastern Iberian Peninsula, and northeastern mountain systems, emerging scarcely in Portugal, and by *B. balearica* that occurs in the Betic deep river valleys and in the Balearic islands. The genetic affinity existent between fifteen populations of *B. sempervirens* was evaluated by seven ISSRs primers. Moreover, leaf morpho-anatomical measurements indicative of leaf performance (such as leaf area, leaf mass per unit area, width, length, shape factor, tissues thickness, stomatal density, and quantification of epi- and intracuticular waxes) and environmental characterization were conducted to get insight into the functional ecology of the genus *Buxus*. Six populations of *B. balearica* and 15 of *B. sempervirens*, collected in the Iberian Peninsula and the Balearic islands, were included to establish the interspecific ranges. A functional ecological description by morpho-anatomical analysis of leaves and a genetic and environmental approach exposed differences between Pyrenaean–Cantabrian–Portuguese and Iberian *B. sempervirens* populations. Genetic relationships among *Buxus* populations were investigated using inter-simple sequence repeat (ISSR) markers. Seven ISSR primers generated a total of 159 unambiguous and repeatable bands, of which 156 (98.1%) were polymorphic. These divergences for Iberian *B. sempervirens* are explained by the isolation in their distributions since the Oligocene. Previsions for future climate change scenarios confirm those biogeographic divergences for the Iberian *B. sempervirens*.

Key words: *B. sempervirens*, genetic flows, molecular marker, leaf anatomy, climatic characterization, Oligocene–Holocene evolution

1. Introduction

Buxus L. is represented in the western Mediterranean basin by evergreen shrubs, usually no more than 5 m tall (Benedí, 1997; Lázaro and Traveset, 2006; Lázaro et al., 2006; Roselló et al., 2007; Di Domenico et al., 2011). Both species are monoecious; the inflorescences contain one female flower surrounded by male flowers (usually four), or in some cases individuals with just male flowers. They are self-compatible and ambophilous (pollinization by insects or wind), and their fruits are dehiscent capsules. Small black seeds are barochory, which explains the concentration of individuals and the access to abiotic means for wider disseminations (Köhler, 2007). This is a genus of exogenous origin that reached Western Europe by glacial dynamics (Di Domenico et al., 2011). Studies of the glacial/interglacial genetic flow between the Iberian Peninsula and the Western Mediterranean areas of several plant species have stated the important concentration of floristic diversity in the Iberian Peninsula, as a result of climatic change's impact on the chorology of unrelated floristic groups, and revealed biogeographic similarities of several taxa (Almeida da Silva et al., 2014). In this context, a better understanding of the natural migration flows from Eastern Europe and the Middle East penetrating the Iberian Peninsula, and their implications on diversification in situ highlighted by the genus *Buxus* are of remarkable interest. The occurrence of this genus in the western Mediterranean basin involves two species, *B. sempervirens* L. and *B. balearica* Lam. (Benedí, 1997). Some authors refer to overlapping for both species in

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northwestern Africa (Tunisia, Algeria, and Morocco) (Leporatti and Ghedira, 2009) and Turkey (Davis, 1988), but no more common occurrences have been reported. *B. sempervirens* is observed along the north, northwestern, and eastern Iberian Peninsula, while *B. balearica* is restricted to Sardinia, Mallorca, the southeastern Iberian Peninsula, northern Morocco, and southern Turkey. This biogeographical characteristic suggests a tertiary divergence between these two species, with current geographic overlap only in the Iberian Peninsula and Turkey (Pignatti, 1978).

It is accepted that coadaptative evolutionary changes in the physiology or morphology of any genus have occurred on a local or regional scale basis, and that consequently populations within a single species may diverge in their characteristics (Ferea et al., 1999). Nevertheless, as responses to changes in environmental conditions occur at different time scales, several degrees of acclimation may result. Leaf gas exchanges rapidly respond to changes in light intensity, temperature, vapor pressure deficit, and leaf water potential, but it can also acclimate to distinct environmental conditions more slowly, as a result of long-term changes in its mass per area (leaf mass per area (LMA)) and leaf chemistry (Brooks et al., 1996). According to Letts et al. (2011), *B. sempervirens* exhibits high compensatory physiological and morphological acclimation to light intensity. Therefore, it is also possible anatomically that leaf modifications resulted from acclimation to water stressed habitats for this species, such as increased mesophyll thickness and density as adaptive responses to stressful conditions (Poorter et al., 2009). Thus the search for leaf anatomical traits indicative of leaf performance seems crucial to get insight into the functional ecology of the genus *Buxus*.

DNA-based markers are suitable tools for a reliable evaluation, characterization, and taxonomical analysis of plants. They provide a good classification of plant species because the genetic material may be directly compared independently of environmental influences (Kumar et al., 2009).

The inter-simple sequence repeats (ISSRs) are considered useful markers in different areas, such as phylogenetics, genetic identity and diversity, gene tagging, genome mapping, and evolutionary biology (Zietkiewicz et al., 1994; Kumar et al., 2009; Sutkowska et al., 2014). ISSRs are known to be abundant, very reproducible, and highly polymorphic, informative, and straightforward to use (Zietkiewicz et al., 1994), especially in similar works with other genera for the Iberian Peninsula (Gómez-Gómez et al., 2012; Rodrigues et al., 2013).

In the present work we employed ISSR markers with the goal of assessing the level and pattern of genetic variability within and among the two species of the genus *Buxus* found in the Iberian Peninsula and Balearic islands to evaluate their taxonomic differentiation and phylogeny. The morpho-genetic differences will be decisive to discuss the recent evolution of both species and their probable biogeographic dynamic during the Late Quaternary period (Di Domenico et al., 2012), i.e. since the fragmentation and separation of their occurrence areas. Several examples with other genera and species are also applied to explain recent morpho-genetic differentiations (Mazur et al., 2010; Fuertes Aguilar et al., 2011). On the other hand, biogeographic dynamics data can help in policy decisions for conservation of the taxa, in particular in the incorporation of *B. sempervirens* into the red list of threatened species.

2. Materials and methods

2.1. Study area and data collection

The distribution of *Buxus* in the Iberian Peninsula and Balearic islands (Figure 1) was determined based on herbaria information. The occurrence of *B. sempervirens* is concentrated along the northeastern mountains and the Cantabrian mountains systems, the Iberian mountains systems, and there is a dispersed population on the western Iberian Peninsula (Portugal and Occident of the Central mountains system). *B. balearica* just occurs in the west of Mallorca (Balearic islands), and occasionally in the eastern mountains and on the Dragonera islet (palynological evidence also indicates the presence of this taxon on the island of Minorca (Yll et al., 1997)), and the southern Betic mountain system (provinces of Málaga and Almeria, Iberian Peninsula), despite the Algerian and Moroccan populations (Lázaro et al., 2006; Navarro-Cerrillo et al., 2011). Twenty-one populations of the two *Buxus* species were selected for the present study. These included 15 populations of *B. sempervirens* from four different regions (four populations from deep valleys of northern Portuguese rivers, six populations from the eastern Iberian Peninsula, three populations from the northeast, and two populations from the Cantabrian mountain system) and six populations of *B. balerica* (three populations from deep river valleys of the Betic mountain system and three populations from the Balearic mountain system) (Table 1; Figure 1). For every population five individuals were collected. These individuals should be distant from each other to guarantee a lack of recent kinship and, at the same time, the morphological variability of individuals was also evaluated. Locations were selected based on information obtained from the following Iberian herbaria: HVR, MA, PO, and SA (designations according to *Index Herbariorum* codes; http://sweetgum.nybg.org/ih/). This previous analysis described the biogeographic distribution of *Buxus* spp. in the Iberian Peninsula, decisive to select the locations for the morpho-genetic analysis.

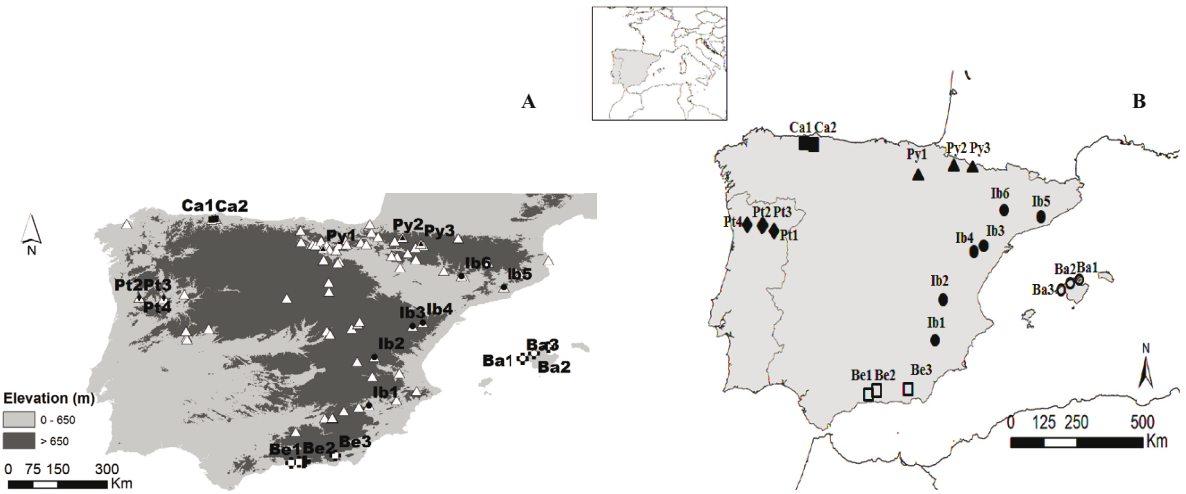

Figure 1. A- Locations of populations of *B. sempervirens* (Δ) and *B. balearica* (✚) in the Iberian Peninsula and Balearic islands) based on the herbaria information, and B- locations of the populations sampled for *B. sempervirens* (●, eastern Iberian Peninsula specimens (Ib); ▲, northeastern mountains specimens (Py); ■, Cantabrian specimens (Ca); ◆, Portuguese specimens (Pt)) and *B. balearica* (○, Balearic specimens (Ba); □, Betic specimens (Be)). Black lines represent the highest density presence areas for *B. sempervirens* in the Iberian Peninsula. The altitudinal classes > 650 m show the areas occupied by the most relevant system mountains of the Iberian Peninsula.

Table 1. Origin of the 21 *Buxus* populations examined (Ba - Balearic mountain system; Be - Betic mountain system; Ca - Cantabrian mountain system; Ib - eastern Iberian Peninsula; Py - northeastern mountain system; Pt - Portugal; * Provinces indicated according to the notation adopted by *Flora Iberica* (http://www.floraiberica.es/generalidades/introduccion.php).

Species	Code	Region	Location*
B. sempervirens	Ib1	Eastern	Mu, El Cenajo (N38.35062° W01.7637°, 477 m)
	Ib2	Eastern	Cu, Enguídanos (N39.67729° W01.61749°, 716 m)
	Ib3	Eastern	Te, Fortanete (N40.51709° W00.57703°, 1438 m)
	Ib4	Eastern	Cs, La Mata de Morella (N40.61367° W00.2955°, 810 m)
	Ib5	Eastern	B, Vacarisses (N41.57931° E01.920331°, 298 m)
	Ib6	Eastern	L, Monestir d´Avellane (N41.88293° E00.758693°, 552 m)
	Py1	Northeastern	Bu, Bugedo (N42.63697° W03.0218°, 568 m)
	Py2	Northeastern	Na, Belagua (N42.93853° W00.85246°, 1174 m)
	Py3	Northeastern	Hu, Sallent de Gállego (N42.76699° W00.34439°, 1399 m)
	Ca1	Cantabrian	O, Agüera (N43.4472° W05.96088°, 206 m)
	Ca2	Cantabrian	O, Grullos (N43.43178° W06.0636°, 72 m)
	Pt1	Portuguese	TM, São Lourenço (N41.2920° W07.3761°, 154 m)
	Pt2	Portuguese	DL, Olo (N41.29687° W08.04433°, 75 m)
	Pt3	Portuguese	DL, Olo II (N41.29624° W08.04729°, 75 m)
	Pt4	Portuguese	DL, Fridão (N41.310104° W08.047246°, 82 m)
B. balearica	Be1	Betic	Ma, Frigiliana (N36.78393° W03.89365°, 249 m)
	Be2	Betic	Gr, Otívar (N36.81727° W03.68049°, 322 m)
	Be3	Betic	Al, Rágol (N36.99302° W02.68647°, 533 m)
	Ba1	Balearic	PM, Formentor (N39.9505°, E03.186797°, 268 m)
	Ba2	Balearic	PM, Cúber (N39.77788° E02.792812°, 756 m)
	Ba3	Balearic	PM, Galatzó (N39.63706° E02.485408°, 825 m)

2.2. Morpho-anatomical analysis

Measurements of leaf morphology included leaf area (LA, cm^2), width, length, and shape factor (SF: the ratio of the actual perimeter to that of a circle with the same area; SF = P/Pc, where P is the perimeter of the object and Pc is the perimeter of a circle with the same area as the object) were conducted with a leaf area meter (WinDias, Delta-T Devices Ltd., Cambridge, UK) in six fully expanded mature leaves randomly collected from each of five adult individuals chosen in each population. Leaves were then dried in a force-draft oven at 70 °C to constant mass. The average leaf mass per area LMA (g m^{-2}) was calculated as the ratio between the leaf dry mass and its total leaf area (LA) according to the procedure proposed by Dijkstra (1989).

Leaves' cross sections were prepared for microscopic examination. Samples from each population were taken from the middle of the leaves, avoiding the differential thickness along the leaf. Sections were fixed in FAA solution (formalin:acetic acid:ethanol 70% 1:1:18), dehydrated in a series of ethanol solutions (70%, 80%, 90%, 100%, 1 h each), and embedded in paraffin. Blocks obtained were sectioned in a manual rotary microtome (Leica RM 2135; Leica Microsystems, Nussloch, Germany) and the sections were stained with 1% toluidine blue O in 0.1 M phosphate buffer (pH 6.8) for 10 min using the method of O'Brien and McCully (1981). The sections were observed using an Olympus IX51 inverted light microscope (Olympus BioSystems, Munich, Germany), photographed with a digital camera (ColorView III; Soft Imaging System GmbH, Münster, Germany), and the image analyzed with the Olympus software Cell. Then the thickness (μm) of the upper cuticle (UC), upper epidermis (UE), palisade parenchyma (PP), spongy parenchyma (SP), PP/SP, lower epidermis (LE), lower cuticle (LC), and total lamina (TL) was measured using Olympus Cell^A software.

Epidermal impressions to assess stomatal density (SD, stomata mm^{-2}) were obtained by applying one or two coats of polish (collodion solution) to the abaxial surface of each leaf. The thin film was then carefully peeled off and placed on a microscope slide. The stomatal density was determined for ten peels obtained from fully expanded leaves collected from the same five individuals in each population used for the other morpho-anatomical measurements.

Soluble cuticular (epi- and intracuticular) waxes were extracted from five samples of 10 mature leaves (from the second year) per population using a modification of the method proposed by Hamilton (1995). After measuring the total LA, samples were immersed in a volume of 50 mL chloroform:methanol (90:10 v:v) solution for 30 s and shaken gently. The extracts obtained were filtered using a Macherey-Nagel (Düren, Germany) MN 713 Filter (0.15 mm thickness) and allowed to evaporate to dryness at room temperature, and then weighed. The quantification of waxes was expressed by the amount of waxes per unit leaf area (μg/cm^2). The leaf morpho-anatomical variables analyzed are shown in Table 2.

Table 2. Morphological traits: leaf area, LA; length, L; width, W; shape factor, SF; perimeter, P; dry mass, DM; leaf mass per unit area, LMA of *B. balearica* (BBaBe), eastern populations (SIb) and Cantabrian– northeastern–Portuguese (SPyCaPt) populations of *B. sempervirens*; anatomical traits (thickness in μm of upper epidermis, UE; palisade parenchyma, PP; spongy parenchyma, SP; lower epidermis, LE; lower cuticle, LC; total lamina, TL; stomatal density, SD (stomata/mm^2); and soluble waxes, SWs (μg/cm^2), of *B. balearica* (BBaBe), eastern populations (SIb), and Cantabrian– northeastern–Portuguese (SPyCaPt) populations of *B. sempervirens* (Pop. = populations; Pval = P-value; numerical values are exposed with their respective deviations, here indicated by ±; values with the same letter for average value (a, b, or c) per population area (BBaBe, SPyCaPt, SIb) and species indicate no significant difference in morphological variable).

Pop.	LA (cm^2)	L (cm)	W (cm)	SF	P (cm)	DM (g)	LMA (g m^{-2})
BBaBe	2.52 ± 1.01c	2.94 ± 0.27c	1.20 ± 0.27c	1.85 ± 0.70b	9.57 ± 2.70b	0.06 ± 0.02b	279.86 ± 117.1c
SPyCaPt	1.74 ± 0.53b	2.41 ± 0.38b	1.08 ± 0.29b	1.69 ± 0.17a	7.76 ± 1.23a	0.03 ± 0.01a	210.70 ± 52.9a
SIb	1.41 ± 0.35a	2.23 ± 0.32a	0.91 ± 0.16a	1.78 ± 0.20b	7.39 ± 1.26a	0.03 ± 0.01a	243.37 ± 55.3b
P val	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

	Thickness (μm)									
	Upper Cuticle	Upper epidermis	Palisade parenchyma (PP)	Spongy parenchyma (SP)	PP/SP	Lower epidermis	Lower cuticle	Total lamina	Stomatal density (stomata/mm^2)	Soluble waxes (μg/cm^2)
BBaBe	14.84 ± 5.57a	18.64 ± 4.96c	265.72 ± 100b	229.01 ± 52.5b	1.15 ± 0.54b	16.00 ± 5.03b	14.91 ± 5.71a	559.12 ± 152b	164.77 ± 20.4c	98.32 ± 66.7a
SPyCaPt	16.45 ± 2.68b	12.51 ± 2.23a	223.60 ± 28.9a	195.06 ± 31.4a	1.19 ± 0.31b	12.43 ± 3.55a	15.08 ± 2.31a	475.13 ± 55.3a	128.83 ± 18.1a	129.49 ± 33.2b
SIb	20.71 ± 1.76c	16.12 ± 3.68b	235.59 ± 65.4a	248.79 ± 54.6c	0.96 ± 0.16a	15.01 ± 3.54b	19.89 ± 2.63b	556.11 ± 128b	154.77 ± 17.0b	175.85 ± 77.6c
P val	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

The above data were analyzed using analysis of variance and, when ANOVA showed significant variable effect (P-value < 0.05), means were separated by Duncan's significant difference test. A forward stepwise discriminant canonical analysis (DCA) for the morpho-anatomical matrix was applied to discriminate the most significant variables that allow distinguishing of the morpho-anatomical behaviors for both species. This capacity to discriminate was based on the F statistic (F-remove) and P-values to describe the distribution of variables. Wilks' lambda was also used to explain variance between variables (1 minus the squared canonical correlation) and their tolerances (in this case 1 minus the squared multiple correlation).

2.3. Molecular analysis

Total genomic DNA was extracted from young leaves of 5 individual plants from each population using the NucleoSpin Plant kit (Macherey-Nagel, Düren, Germany). Out of the 19 ISSR primers (UBC#100/9) tested, seven were selected on the basis of the high polymorphism revealed. The PCR amplifications were performed in a total volume of 20 µL with Taq polymerase enzyme and the protocol adapted from Zietkiewicz et al. (1994). The program of amplification reactions involved an initial denaturation at 94 °C for 5 min followed by 45 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplification products were run on 1.7% agarose gels stained with ethidium bromide. To determine the number of polymorphic, exclusive, and total bands and the polymorphism percentage only clear and distinct bands were scored. A binary matrix based on the molecular marker dataset was derived taking into account the presence and absence of a band. The ISSR data were analyzed using the Popgene 1.32 software and multiple populations were selected as an input option in the initial menu of this software. This setting enables one to calculate the following parameters: Shannon's information index (which measures gene diversity (Shannon and Weaver, 1949)), Nei's gene diversity index (h = 1 – Ppi 2, where pi is the frequency of the ith allele of the locus (Nei, 1978)), total genetic diversity (H_T), genetic diversity within groups (H_S), and relative magnitude of differentiation among groups (G_{ST}). The information content of each marker system was determined according to the indices of Powell et al. (1996): effective multiplex ratio (EMR) (number of polymorphic products from a single amplification reaction) and marker index (MI) (product of effective multiplex ratio). According to Prevost and Wilkinson (1999), the ability of the markers to differentiate the species/varieties was assessed by calculating their resolving power (Rp) using the formula Rp = PIb, where Ib = 1 – [2 × (0.5 – p_i)] and p_i is the proportion of the plants containing the I band. The polymorphic index content (PIC) was obtained using the formula:

$$PIC = 1 - \sum_{i=1}^{i} P^2\, i - \sum_{i=1}^{i-1} \sum_{j=i+1}^{i} 2P_i^2\, P_j^2$$

where Pi and Pj are the frequencies of the ith and jth alleles, calculated using the software PICcalc. Analysis of molecular variance (AMOVA) was performed using the GenAlEx 6 software (Peakall and Smouse, 2006) in order to partition the genetic variation between species and populations and within populations (Schneider et al., 2000). The F-statistic was estimated using a Bayesian approach with the Φ-statistic, an analogue of the F-statistic. The parameter Φst is more correct once it incorporates the genetic distances among alleles (Weir and Cockerham, 1984). The significance of each variance component was tested with permutation tests (Excoffier et al., 1992). Genetic distances were estimated according to Nei (1978) and principal coordinate analysis (PCoA) was performed. Genetic similarity matrices based on Jacard and neighbor-joining cluster analyses were used to construct genetic trees using Darwin software. Bootstrap analysis was used to verify if the number of polymorphic loci evaluated was high enough to provide accurate genetic distance estimates. To determine the sampling variance of the genetic distances produced by the different molecular data sets we performed bootstrap analysis using a decreasing number of bands. For each specific number of loci or bands used the polymorphic markers were submitted to 10,000 random samplings with replacement (bootstrap samples) and genetic distances were obtained for each bootstrap sample (Tivang et al., 1994). Species structure and identification of admixed individuals was performed using the model-based software program STRUCTURE 2.3 (Pritchard et al., 2000). This software uses a Markov chain Monte Carlo (MCMC) algorithm to cluster individuals into populations on the basis of multilocus genotype data (Pritchard et al., 2000). The number of populations (K) was estimated by performing at least five runs of STRUCTURE, using 1,000,000 MCMC repetitions and 50,000 burn-in periods by setting K from 1 to 8. Any prior information about the population of origin was used, and correlated allele frequencies and admixture were assumed. The average of the log-likelihood estimates for each K was calculated. The ad hoc statistic ΔK (Evanno et al., 2005) was used to set the number of populations.

2.4. Environmental characterization and modeling distribution and predictions

The environmental values employed in the present work for each location were obtained from the WORLDCLIM

layers (http://www.worldclim.org). The environmental parameters may be found at http://www.worldclim.org/formats.

The morpho-genetic analysis was submitted to environmental (climatic and altitudinal) characterization, as well as future climate change scenarios. The environmental approach was exposed by a principal component and classification analysis (PCCA), using STATISTICA 9.1 (Statsoft Ltd.). Two scenarios for the year 2080 were selected. The climate predictors used were derived from a general circulation model (CCCMA:CGCM2), under IPCC emission scenarios (SRES; A2a and B2a) for the prediction of future potential habitats (http://gisweb.ciat.cgiar.org/GCMPage; Ramirez and Jarvis, 2008). A2a and B2a represent two scenarios with different greenhouse gas emissions: while A2a describes a highly heterogeneous future world with regionally oriented economies, B2a, also regionally oriented, implies a general evolution towards environmental protection (B2a has a lower rate of global warming, and therefore changes in temperature and precipitation are less intense than in A2a) (http://forest.jrc.ec.europa.eu/climate-change/future-trends). Ten repetitions with cross-validation, regularization multiplier of 1 and 500 iterations (Phillips et al., 2006), were elaborated. The obtained output (in ASCII format) was input into ArcGIS software version 9.2 (ESRI, Redlands, CA, USA) as floating-point grids (Peterson et al., 2007) and occurrence probability of species at each site was mapped.

The environmental variables considered as potential predictors for *Buxus* current habitat distribution were chosen based on their biological relevance to plant species distributions and other habitat modeling studies (Murienne et al., 2009). Elevation, monthly precipitation, monthly maximum and minimum temperature, and 19 bioclimatic derived variables biologically most meaningful to define eco-physiological tolerances of a species (Murienne et al., 2009) were obtained from WorldClim (http://www.worldclim.org). With exception of elevation, all variables were obtained from 1950–2000 averaged bioclimatic data. The distribution of *Buxus* was mapped based on the occurrences geographically referenced, using a grid with resolution of 1 × 1 km according to the pixel resolution of the environmental variables used. Less precise occurrence data (more than a square kilometer) were not used, although location errors up to 5 km appear to have no impact on model performance (Graham et al., 2008). Testing or validating the fit or accuracy of the modeling approach, as well as determining the probability that a presence location will be ranked higher than a random background location, was done through ROC plots and AUC approaches (Phillips et al., 2006). These random background locations serve as pseudo-absences for all analysis in Maxent (Phillips et al., 2006).

3. Results

3.1. Morpho-anatomical analysis

Buxus balearica and *B. sempervirens* leaves are dorsiventral and hypostomatic and reveal xeromorphic features, as demonstrated by a thick cuticle and a compact and thick mesophyll. Differences between the leaves of the studied populations were detected (Figures 2A–2C; Table 2). Cantabrian, northeastern, and Portuguese mountain systems *B. sempervirens* have thicker leaves (475 μm) than Iberian mountain system populations (556 μm) and *B. balearica* populations (559 μm). Moreover, *B. balearica* leaves present higher LA (2.5 cm^2), LMA (279 g m^{-2}), and stomatal density (SD) (165 stomata mm^{-2}), but lower content of soluble epicuticular waxes (SWs) (98 μg cm^{-2}) than *B. sempervirens* leaves (129.49 μg cm^{-2} and 175.85 μg cm^{-2}, in the Cantabrian–Portuguese system and eastern Iberian Peninsula, respectively). Among the *B. sempervirens* species, the eastern populations have wider upper epidermis (UE) and higher SW content than the specimens from the Cantabrian and northeastern mountain systems and from Portugal.

As expected, the DCA applied on the morpho-anatomical parameters shows greater relevance to distinguish *B. sempervirens* from *B. balearica* (Figure 3). The UC thickness is the principal morpho-anatomical variable that discriminates all the populations (Table 3), accounting for 76% of the total variance (F-remove = 76.082, Wilks' lambda = 0.344, P-value = <0.001). The first component reveals significant differences between *B. balearica* and *B. sempervirens* populations. The second component shows two morpho-anatomic tendencies for *B. sempervirens* populations: the northern (Cantabrian, northeastern, and Portuguese) populations from the eastern mountain system.

3.2. Molecular analysis

The seven primers produced a total of 159 scorable markers among the 21 populations investigated. The size of the amplified products ranged from 200 to 3000 bp. The number of markers produced per primer ranged from 15 to 30. The total number of polymorphic markers and the percentage of polymorphism were 156 and 98.1, respectively (Table 4). Out of the 156 polymorphic bands, nine were considered specific. These results indicate that only 5.8% of the bands could be considered population-specific. The PIC average was 0.34, the highest value (0.39) being observed in primer UBC808 and the lowest value (0.29) with primer UBC810. The Rp, EMR, and MI average values were 17.21, 21.90, and 33.22, respectively. Primer UBC859 exhibited the highest Rp (22.67), whereas the lowest Rp (11.330) was found for primer UBC811. The highest values of MI and EMR were observed with primer UBC808 (38.55 and 26.00, respectively). Primer UBC823 led to the lowest values of MI and EMR (27.83 and 11.27,

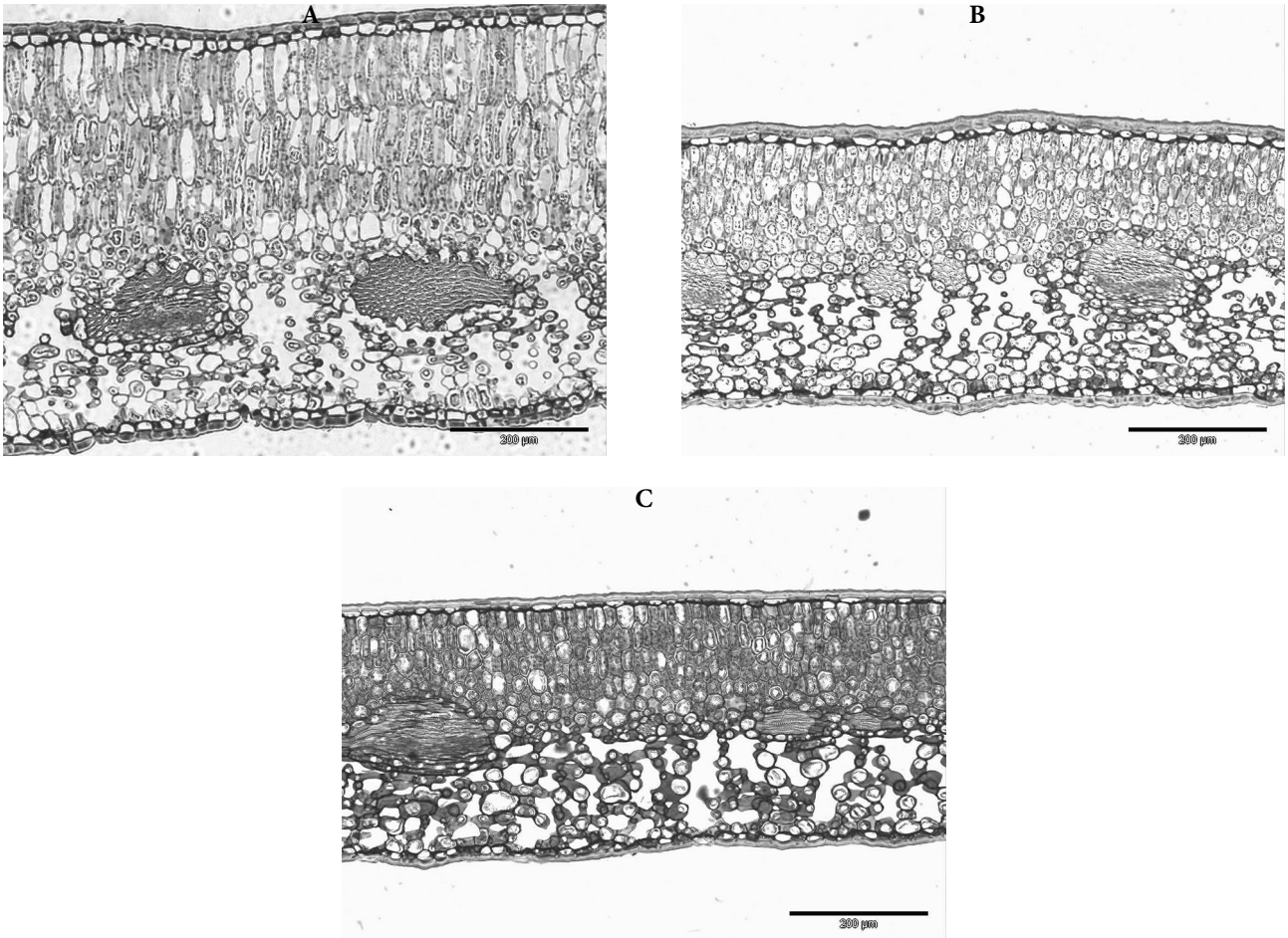

Figure 2. A- Leaf cross sections of *B. balearica*. B- for the Iberian mountain system populations. C- and for Cantabrian–northeastern–Portuguese populations of *B. sempervirens*.

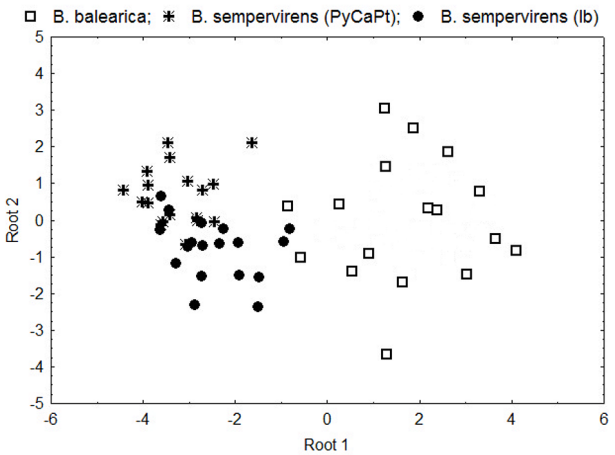

Figure 3. DCA of anatomical parameters of *B. balearica*, eastern populations of *B. sempervirens* (Ib) and Cantabrian–northeastern–Portuguese populations of *B. sempervirens* (PyCaPt).

Table 3. Numerical values for the DCA upon the morpho-anatomical matrix for *B. balearica* and both groups of *B. sempervirens* (upper cuticle, UC; spongy parenchyma, SP; lower cuticle, LC; total lamina, TL).

Variables	Wilks' lambda	F-remove	P-value	Toler.
UC	0.344222	76.08234	<0.001	0.83882
LC	0.161094	13.26209	<0.001	0.81063
SP	0.146757	8.34373	0.001	0.42495
TL	0.127698	1.80582	0.17066	0.3506

Table 4. Inter-simple sequence repeat polymorphism generated in the 21 populations of *B. sempervirens* and *B. balearica*.

Primer	TNB	NPB	NEB	P%	Rp	PIC	MI	EMR
808	26	26	1	100	19.710	0.390	38.550	26.000
810	23	23	4	100	13.620	0.290	29.030	23.000
811	22	22	1	100	11.330	0.296	29.560	22.000
823	15	13	1	86.667	14.760	0.320	27.830	11.267
855	18	18	1	100	16.860	0.370	36.630	18.000
859	25	24	1	96.000	22.670	0.350	33.260	23.040
880	30	30	0	100	21.520	0.380	37.700	30.000
Total	159	156	9		120.470		232.560	153.307
Average	22.714	22.286	1.286	97.524	17.210	0.342	33.223	21.901

TNB, Total number of bands; NPB, Number of polymorphic bands; NEB, Number of exclusive bands; P%, Polymorphism percentage; Rp, Resolution Power; PIC, Polymorphism Information Content; MI, Marker Index; and EMR, Effective Multiplex Ratio.

respectively). For the 21 populations, the number of alleles, effective number of alleles, Nei's genetic diversity, and Shannon's information index were 1.982, 1.556, 0.33, and 0.47, respectively. The values for the total genetic diversity among groups (Ht) and within groups (Hs) were 0.33 and 0.08, respectively. The value obtained for the mean coefficient of gene differentiation (Gst) (0.77) indicates that 23% of the genetic diversity resides within the populations. The estimated gene flow in the populations was 0.15 (Table 5). An UPGMA dendrogram based on Dice distance was generated in order to depict relationships among the 21 populations studied (Figure 4). The Dice coefficient of similarity ranged from 0.98 to 0.25. The dendrogram clearly separates the two species of *Buxus* and shows a good tendency of the populations to cluster according to their geographic origin. Cluster B contains all the populations of *B. balearica* and cluster S includes the 15 populations of *B. sempervirens*. In cluster B, *B. balearica* populations are divided in two independent subclusters, one comprising the populations of the Betic mountain system and the other one including the Balearic populations. Cluster S, entirely ascribed to *B. sempervirens* populations, gives rise to a first subcluster that includes practically all the populations of the eastern Iberian Peninsula and a second subcluster containing the populations from the Cantabrian and northeastern mountain systems, from the deep valleys of northern Portuguese rivers and one population from the eastern Iberian Peninsula.

The PCoA showed that the three most informative principal coordinates explained 65.7% of the total variability. The first coordinate accounts for 34.82% of the total variation. It allows the identification of the two species, *B. balearica*, which comprises Balearic and Betic populations, and *B. sempervirens*, which include Cantabrian, northeastern and eastern Iberian, and Portugal populations. The second coordinate explains

Table 5. Analysis of molecular variance for 21 populations of *B. sempervirens* and *B. balearica* (AMOVA, Excoffier et al., 1992).

	na	ne	h	I	H_T	H_S	G_{ST}	Nm
Mean	1.982	1.556	0.330	0.469	0.330	0.076	0.768	0.151
SD	±0.134	±0.307	±0.143	±0.180	±0.020	±0.003		

na-number of alleles, ne-effective number of alleles, h-Nei's (1978) gene diversity index, I-Shannon's information index, H_T-total genetic diversity, H_S-intrapopulation genetic diversity, G_{ST}-relative magnitude of differentiation among groups, Nm-estimate of gene flow from G_{ST}.

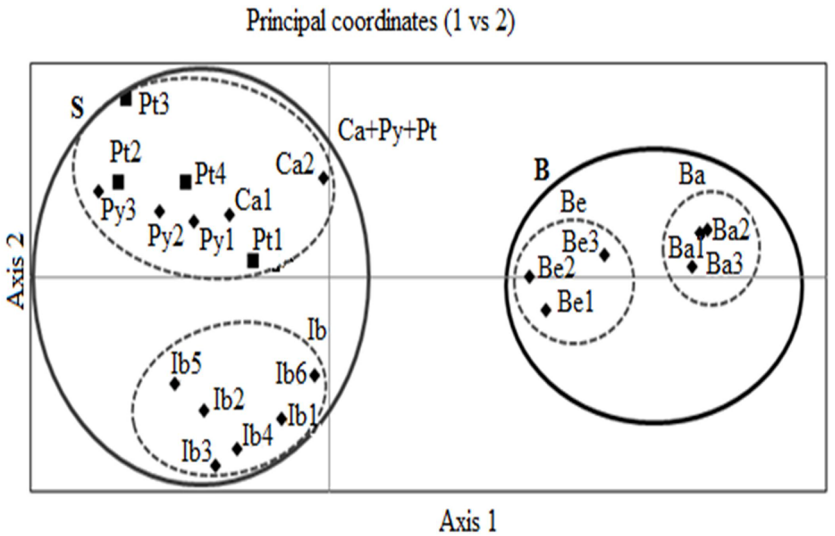

Figure 4. PCoA of twenty-one populations of *Buxus* spp. examined of *B. balearica* (B) and *B. sempervirens* (S).

16.23% of the total variation and splits *B. sempervirens* species two subgroups, the eastern Iberian populations and the remaining populations (Cantabrian, northeastern, and Portuguese) (Figure 5). The result of PCoA is in agreement with the hierarchical analysis. The AMOVA, performed with the 21 populations of *Buxus* spp., led to a Φ_{PT} value of genetic variation between species of 0.26 (P-value < 0.00), which indicates a very great genetic differentiation (Table 5). Molecular variance was 26% among species and within species was 74%, indicating that there is more variation within species than among species.

The 21 *Buxus* populations were further evaluated for population stratification using the STRUCTURE software (Pritchard et al., 2000). ISSR data were analyzed increasing the number of subpopulations (K) from 1 to 8. The estimation of ΔK revealed the highest value for K = 2 (ΔK = –8565.58), splitting the populations into two groups corresponding to *B. sempervirens* (group I) and *B. balearica* (group II) (Figure 5). Group I is formed by 15 populations belonging to *B. sempervirens* and group II is composed of the six populations from *B. balearica*.

3.3. Environmental characterization and modeling distribution and predictions

The analysis of the environmental matrix for these two taxa, obtained by PCCA, shows three different groups (Figure 6), distinguished by mean altitude (alt), diurnal range, isothermality, temperature seasonality, temperature of the wettest quarter, temperature of the driest quarter, annual precipitation, precipitation in June, precipitation in September, and temperature in November (Table 6): one environmental group for *B. balearica* (higher mean temperature of driest quarter, lower mean precipitation in June, lower mean precipitation in September, and mean maximum temperature in November), and two different environmental groups for *B. sempervirens*. The latter two groups for *B. sempervirens* represent the specimens restricted to the east of the Iberian Peninsula (higher average altitude, higher mean diurnal range, higher mean temperature seasonality (annual variation of temperature), higher mean temperature of wettest quarter, lower mean temperature of driest month, and lower mean maximum temperature in November). On the other hand,

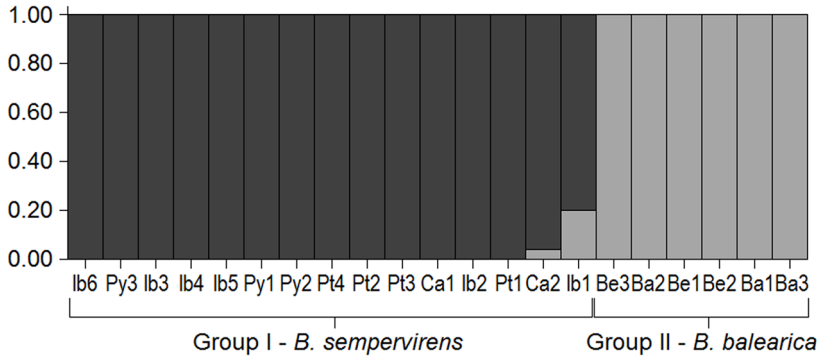

Figure 5. Group assignment of the twenty-one populations of *Buxus* spp. Each individual bar represents a population.

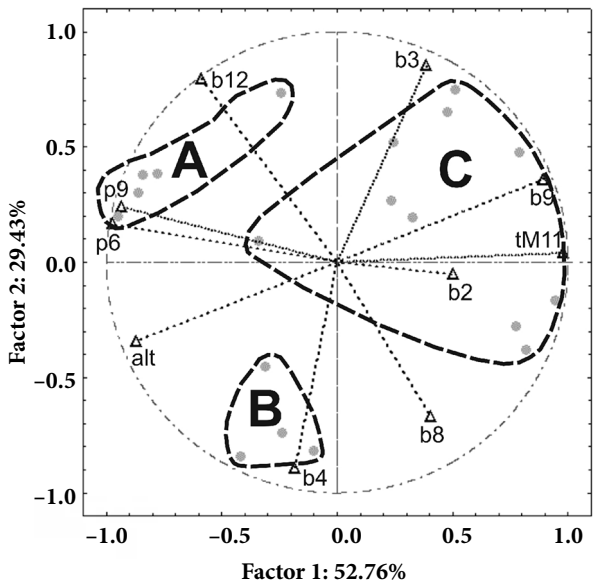

Figure 6. PCCA of the environmental matrix for populations of *Buxus* spp. examined. Three environmental groups are obtained: A- environmental group for *B. balearica*, B- environmental group for Iberian system mountains *B. sempervirens*; and C- environmental group for northeastern–Cantabrian–Portuguese *B. sempervirens* (b2, mean diurnal range or monthly differentiation between highest and lowest temperature; b3, isothermality (coefficient between b2 and annual range temperature × 100); b4, temperature seasonality (standard deviation × 100); b8, mean temperature of wettest quarter; b9, mean temperature of driest quarter; b12, annual precipitation; tM11, average monthly minimum temperature of November (°C × 10); p6, average monthly precipitation of June (mm); p9, average monthly precipitation of Setember (mm); alt, altitude (m)).

the specimens from the Cantabrian and northeastern mountain systems and from Portugal are characterized by higher isothermality, lower mean temperature seasonality, lower mean temperature of the wettest quarter, higher annual precipitation, and higher mean precipitation in June and September. The potential distribution area for *B. sempervirens* is reproduced in Figure 7A. Their modeling previsions maps in Figures 7B and 7C (for the eastern Iberian Peninsula) and Figures 7D and 7E (Cantabrian–northeastern–Portuguese) for future climate change scenarios B2 and A2 in 2080 are represented. These previsions suggest very important alterations in the relevance of *B. sempervirens* in the Iberian Peninsula. This species experiences a very conspicuous decrease in the light of the above scenarios. This effect is particularly evident for the Cantabrian–northeastern–Portuguese group, with more significant decreases for A2 scenarios (in this case *B. sempervirens* is just restricted to the Pyrenees mountain systems and small areas along the Cantabrian mountains). Similar responses are obtained for the eastern Iberian mountain system populations.

Table 6. Statistical description of the most discriminant environmental variables describing the potential distributions of *B. balearica*, and both groups of *B. sempervirens* populations (alt, altitude (m); bio2, mean diurnal range (°C × 10); bio3, isothermality (°C × 10); bio4, temperature seasonality (°C × 10); bio8, mean temperature of wettest quarter (°C × 10); bio9, mean temperature of driest quarter (°C × 10); bio12, annual precipitation (mm); p6, average monthly precipitation in June (mm); p9, average monthly precipitation in September (mm); and tmax11, average monthly maximum temperature in November (°C × 10)).

	Mean	Median	Minimum	Maximum	Std. Dev.
B. balearica					
alt	458,333	451,000	142,000	743,000	238,703
bio2	87,500	86,500	76,000	104,000	12,582
bio3	36,000	36,000	34,000	38,000	1,897
bio4	5,326,000	5,275,000	5,113,000	5,575,000	172,408
bio8	114,833	110,500	105,000	139,000	12,561
bio9	223,500	230,000	199,000	242,000	18,609
bio12	546,333	553,000	316,000	762,000	196,970
prec6	19,500	17,000	10,000	32,000	10,521
prec9	41,000	41,000	14,000	69,000	28,164
tmax11	162,333	170,500	138,000	182,000	18,641
B. sempervirens eastern mountain system					
alt	744,500	692,000	324,000	1,397,000	384,553
bio2	99,167	98,000	73,000	118,000	16,510
bio3	36,333	36,000	32,000	40,000	2,658
bio4	5,980,667	6,037,500	5,419,000	6,304,000	315,797
bio8	142,333	140,000	105,000	198,000	31,207
bio9	107,167	67,500	17,000	238,000	92,636
bio12	529,500	559,500	344,000	629,000	114,516
prec6	51,167	52,000	23,000	67,000	16,266
prec9	54,333	60,000	28,000	74,000	16,765
tmax11	131,333	128,000	92,000	173,000	27,391
B. sempervirens Cantabrian–northeastern–Portuguese					
alt	460,889	206,000	35,000	1,408,000	531,340
bio2	95,667	95,000	83,000	108,000	8,307
bio3	40,889	42,000	38,000	44,000	2,205
bio4	4,819,111	4,759,000	3,821,000	5,609,000	631,375
bio8	87,000	99,000	18,000	122,000	30,574
bio9	186,000	189,000	133,000	215,000	31,040
bio12	955,000	1,041,000	729,000	1,098,000	130,457
prec6	56,889	47,000	40,000	97,000	22,790
prec9	62,444	60,000	46,000	84,000	12,905
tmax11	133,444	150,000	70,000	162,000	36,586

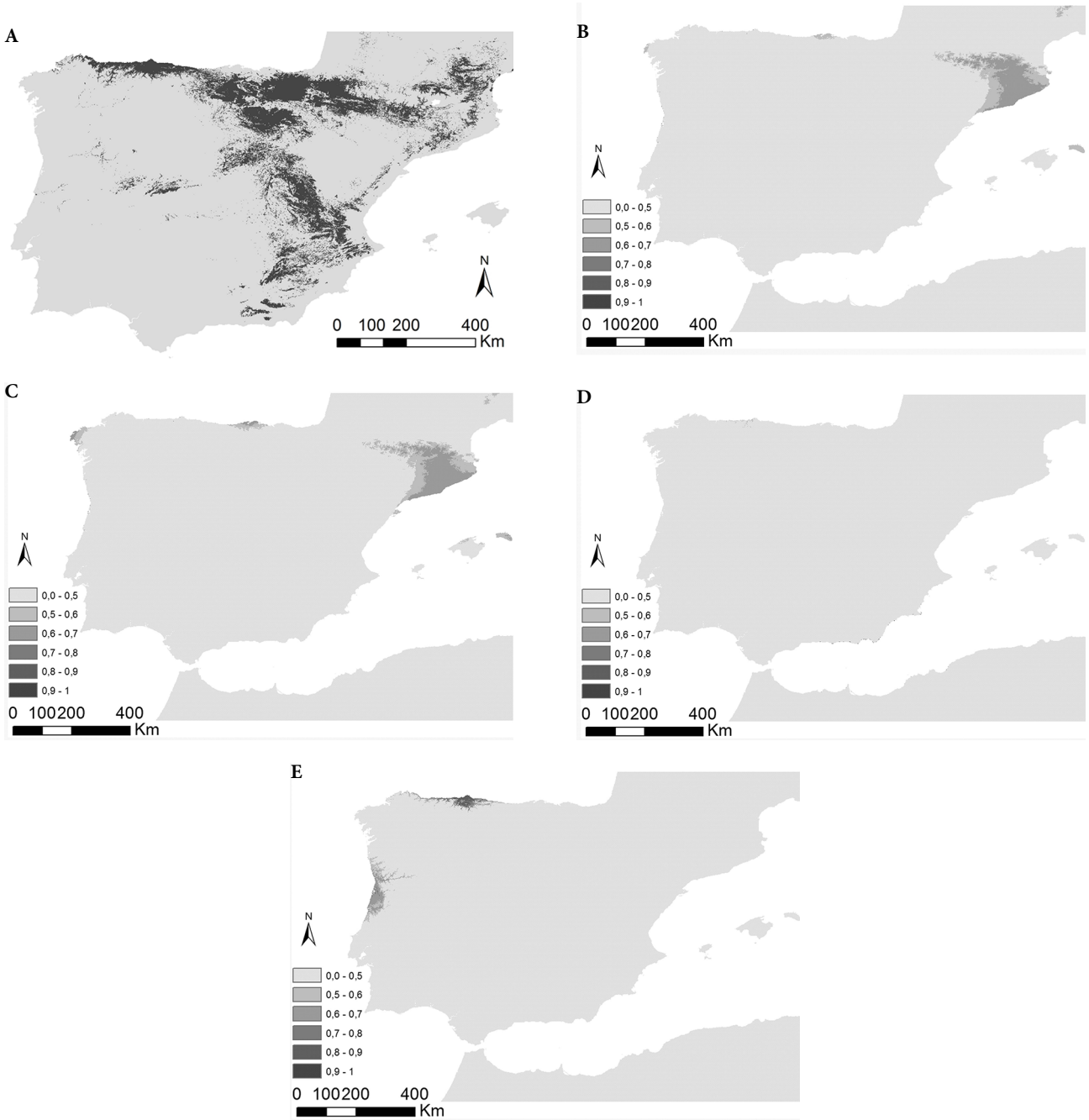

Figure 7. Potential and predictable areas for species and environmental groups for *B. sempervirens*: A- potential area for the species, B- potential predictable area for the eastern Iberian Peninsula environmental group of *B. sempervirens* in climate change scenarios Ba2, and C- Aa2 for 2080, D- potential predictable area for the northeastern–Cantabrian–Portuguese environmental group of *B. sempervirens* in scenarios Ba2, and E- Aa2 for 2080.

4. Discussion

Buxus, the largest genus in the family Buxaceae (Wang et al., 2012), is represented in the Iberian Peninsula and the Mediterranean basin by *B. sempervirens* (in the most temperate areas) and *B. balearica* (in warmer and humid zones). The presence of *Buxus* species in the Iberian Peninsula must be involved in the floristic transformation process of the Mediterranean basin from Oligocene to Pliocene (Postigo Mijarra et al., 2009; Jiménez-Moreno et al., 2010). A biogeographic pattern like this distinguished the temperate and mountain *B. sempervirens* from the warmer climate distribution of *B. balearica*, located

in the unglaciated refugia of the Mediterranean basin and northwestern Africa (Roselló et al., 2007). This current biogeographic divergence could be explained as an adaptation to the increasing dryness during the Miocene (Di Domenico et al., 2011) and their more recent reorganization in the Holo-Pleistocene period (Schönswetter et al., 2005) (here with more incidence on *B. sempervirens*, as supported by other authors for mountain species (Schönswetter et al., 2005)).

The functional ecology is described by the morpho-anatomical results. Leaves of both species are xeromorphic due to the very thick cuticle over both the upper and lower epidermis cells. This characteristic could be a mechanism adopted by the leaves to conserve water. The cuticle restricts water vapor loss by forming a hydrophobic barrier, this being the simplest method to conserve water since cuticle biosynthesis is energetically inexpensive (Pallardy, 1981). Nevertheless, *B. balearica* leaves have lower content of SWs than *B. sempervirens*, probably because of the lower need to conserve water in a maritime climate. Among the *B. sempervirens* species group, the eastern Iberian mountain populations are better adapted to drought than the Cantabrian mountain system populations, as confirmed by their thicker UE and higher content of SWs. Moreover, leaves from eastern Iberian mountain populations present higher lamina, higher LMA, and lower LA. Our morpho-anatomical data for both species are thus in perfect agreement with Björkman's (1981) study, which indicates that plants grown under high light generally have thick leaves with a high LMA. Moreover, leaves developing during drought usually have a higher LMA than leaves subjected to well-watered conditions in Mediterranean species such as in *Quercus ilex* (Salleo and Lo Gullo, 1990) and in *Olea europaea* (Bacelar et al., 2004). A high LMA is usually a consequence of an increase in density or thickness of foliar tissue and normally occurs when the costs of the assimilatory apparatus increase (Centritto, 2002), such as during long periods of drought. Furthermore, eastern Iberian mountains *B. sempervirens* populations present higher SD to probably avoid water stress through flexible regulation (Bolhar-Nordenkampf, 1987).

On account of the high difficulty in distinguishing *Buxus* species, studies using molecular techniques have been used to supplement morphological analysis with the aim to establish the relationship between species and understand their evolution (Wang et al., 2012). To date, only a few studies of *Buxus* based on DNA-based markers have been performed. Savesen et al. (2009) demonstrated, on the basis of biometric analysis and AFLP markers, that 3 morphologies of *B. sempervirens*, assumed to have represented genetically distinct old cultivars of the 17th or 18th centuries, are morphologically and genetically different. Jiang et al. (2008) and Ly and Ji (2009) evaluated the relationships among clones of *B. sinica* var. *parvifolia* and obtained 85.9% and 82.4% of polymorphism by RAPD and ISSR, respectively. Wang et al. (2012) analyzed later rDNA ITS sequences in different species of *Buxus*, with remarkable results to describe genetic relationships between *B. sinica* var. *parviflora* and *B. henryi*. The molecular approach with ISSR markers confirms genetic differences between *B. sempervirens* populations from the eastern Iberian Peninsula and the others from the Cantabrian–northeastern–Portuguese locations. This genetic divergence was even more remarkable than that observed for *B. balearica* from Balearic or southwestern Iberian populations. Finally, the environmental analysis also confirmed these divergences for *B. sempervirens*, with two biogeographic behaviors: a more humid and temperate north and western distribution (Cantabrian–northeastern–Portuguese) and a dryer and warmer eastern one (eastern Iberian Peninsula).

The geotectonical dynamic of the occidental Mediterranean basin over the last 30 million years (Viti et al., 2009) and the glacial–interglacial floristic phenomenon have deeply influenced the evolution of the Iberian Peninsula and Balearic islands vegetation (Pignatti, 1978; Postigo Mijarra et al., 2009). This environmental process provides the biogeographic advance of flora from northern to southern and the existence of micro refuges (Di Domenico et al., 2012) and reinforces the existence of two different biogeographic dynamics, one occident and the other oriental (Rodríguez-Sánchez et al., 2010), during the glacial periods. In contrast, opposite dynamics from south to north occurred during the interglacial periods, as described on the western side of the Iberian Peninsula and northwestern Morocco (Almeida da Silva et al., 2014), limited on the eastern side by the biological Almeria border (Hernández Bermejo and Saínz Ollero, 1984). Based on these descriptions about recent patterns of flora in the Iberian Peninsula, two different biogeographic routes are observed: one from the Pyrenees westward along the Cantabrian Mountains system and the other by the eastern mountains system with access to the Central Mountains of the Iberian Peninsula. This biogeographic divergence associated with glacial-interglacial dynamics, as well as the climate transition in a heterogeneous topographic environment (Cowling et al., 1996), will explain the high concentration of taxonomic diversity in the Iberian Peninsula and northwestern Africa, hotspot of the Mediterranean basin (Myers et al., 2000). At the same time, this phenomenon will also explain the genetic differences for *B. sempervirens* populations in the Iberian Peninsula, and the occurrence of a temperate species (*B. sempervirens*) along the eastern mountain systems and its fragmentation in low altitude Portuguese populations (Pt1, Pt2, Pt3, Pt4), possible glacial micro refuge areas.

The morpho-anatomical and genetic differences observed between Cantabrian–northeastern–Portuguese *B. sempervirens* populations and the eastern Iberian Peninsula population for this species have been also described for other genera and species, especially as a result of the Oligocene–Pleistocene–Holocenic climate changes (Rodríguez-Sánchez et al., 2010). The North Atlantic Oscillation (NAO) during that period (Pérez Obiol et al., 2010) and the consequent mediterranization involved in this process (Sadori et al., 2011) have been pointed out as the main reasons to distinguish those bioclimatic differences since that period.

The results obtained for modeling environmental previsions alert us to a very substantial change in the occurrence of *B. sempervirens* in the Iberian Peninsula. These previsions also corroborate the biogeographic divergences of this species for the Iberian Peninsula. Almost extinction is expected for Cantabrian–northeastern–Portuguese *B. sempervirens*. This decrease is not so serious for the eastern mountains, but very important reductions in its potential occurrence are also expected. That environmental circumstance is correlated with the morpho-genetic differentiation here observed. This circumstance is really critical, not just for the important decrease in *B. sempervirens*, but also for the disappearance of Cantabrian–northeastern–Portuguese populations, and the total extinction of this species in Portugal in the near future. The conservation of this species is also discussed in northern France, where human intervention is suggested but their occurrence around medieval constructions (Decocq et al., 2004) does not allow it. In contrast, the scarce presence of this taxon in eastern involves it in conservation processes of their vegetal communities (Barbaro et al., 2001). A similar discussion emerged for Turkish or Italian communities with this species (Kaya and Raynal, 2001; Selvi, 2007). In the present case, the Portuguese populations of *B. sempervirens* are not associated with anthropic activities, according to pre-Holocenic germoplasm reported from fossil woods information (Di Domenico et al., 2012); their occurrence exclusively along the riverside gallery forest communities (in the habitat 91B0 (Habitats Directive 92/43/CEE)), or at casmophyte formations on the bankfull area of these rivers; and the genetic similarity of these populations with the northern and northeastern ones. Based on the results, the legal inclusion of this taxon as a threatened species and its consequent preservation are suggested here.

In conclusion, morpho-anatomical, ISSR markers, and environmental characterizations revealed two different genetic flows for *B. sempervirens*. Glacial–interglacial recent processes are involved in the dynamic of *B. sempervirens* along two different paths or genetic flows: Cantabrian–northeastern–Portuguese and eastern mountains. Two important issues emerged from this study and they deserve careful consideration: morpho-genetic divergences undergone by *Buxus* and the biogeographic Iberian divergences reported for several genera. Based on the restrictive presence of *B. sempervirens* (Cantabrian–northeastern–Portuguese populations) in Portugal (restricted to small and isolated areas along a short stretch of the Douro river, and some tributaries), and the previsions obtained under climate change scenarios, the authors suggest the possibility of including this species in the Portuguese vascular plant red list.

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