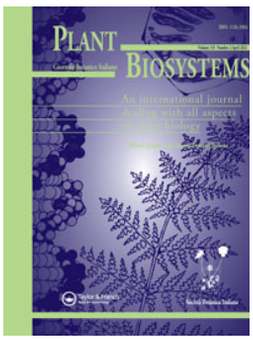

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Limonium mucronatum: plant communities and cytogenetic characterization of an endemic of the Moroccan Atlantic Coast

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ABSTRACT

Limonium mucronatum (L.f.) Chaz. (Plumbaginaceae), a strict endemic of Morocco, has a restricted range in the littoral zone between Rabat and Tan Tan (Draa valley). This coastal region at the junction of Mediterranean, Canary and Saharan influences is of considerable biogeographical interest. However, little information exists on *L. mucronatum* communities and its relationship with bioclimatology or chromosome polymorphisms, which may have an impact in the context of plant conservation. In this study, we analysed *L. mucronatum* communities and performed a cytometric and karyological characterization of this rare species. Results showed two plant communities associated with this species related to a latitudinal and bioclimatic gradient along the surveyed region. Our study provided for the first time new data regarding nuclear DNA amount and about the numbers, positions and organization of 45S rDNA loci in *L. mucronatum*. Remarkably, cytogenetic analyses revealed homogeneous ploidy across all studied populations with all individuals with $2n = 12$ chromosomes. The results obtained are discussed in the context of the originality of flora and the threats to *L. mucronatum* communities, and in the perspective of a conservation strategy for this endemic species.

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Atlantic coast of Morocco; chromosome; ecology; flora; FISH; genome size; plant conservation; vegetation

Introduction

Morocco has the richest flora in North Africa (Médail and Quézel 1997) with circa 4000 species, 426 type subspecies (autonyms) and 872 additional subspecies, and a high percentage of endemic species (22%), with about 920 endemic taxa (Fennane and Ibn Tattou 2012). Two (High and Middle Atlas; Baetic-Rifan complex) of the 10 regional hotspots of Mediterranean biodiversity (Médail and Diadema 2009) are found in Morocco. Its geographical position, integrated into the Mediterranean region, exposed to the Atlantic Ocean and Saharan climatic influences as well as varied geology and topology, largely contributes to the richness of its flora (Benabid 1985, 2000). During the Miocene, Morocco served as a corridor for flora and fauna, and in the Messinian Salinity Crisis (5.96–5.33 Ma) (Duggen et al. 2003), the Gilbraltar strait linked Europe and North Africa as land bridges (Raven 1973; Quézel 1978, 1983; Médail and Myers 2004). This has resulted in rich plant diversity of different biogeographic origins (Palaearctic, Afrotropical) and of great value for conservation (Quézel 1978, 1983; Fennane and Ibn Tattou 1998).

Recently, the practical flora of Morocco (Fennane et al. 1999, 2007, 2014) and an updated checklist of endemic Moroccan flora following the APG III classification were published (Rankou et al. 2013) together with a red list of the endemic monocotyledons of Moroccan flora (Rankou et al. 2015; Fennane 2016, 2017a, 2017b, 2017c, 2018a, 2018b, 2018c, 2018d, 2018e). Although these studies provide valuable and important information on Moroccan flora, there is still limited information on the biodiversity hotspots and the distribution of rare and endemic species. Among the Plumbaginaceae, the genus *Limonium* comprises many annual and perennial taxa found in cliff-tops, rocky and sandy seashores, and saltmarshes (Sauvage and Vindt 1954; van der Maarel and van der Maarel-Versluys 1996; Fennane et al. 1999), including 12 taxa strictly endemic of Morocco (Fennane and Ibn Tattou 1998; Rankou et al. 2013). Among these endemic species, *Limonium mucronatum* (L.f.) Chaz. (Synonym: *Statice mucronata* L.fil.) occurs in about 15 locations, mainly on rocks and coastal sands under subhumid, semi-arid and arid bioclimates, from Rabat to Tan Tan (Draa valley) (Fennane and Ibn Tattou 2005). These Moroccan Atlantic coasts are among the most fragile ecosystems in the country, threatened by the high and increasing population

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density and the expansion of tourist and urban development (Laouina and Berriane 2005). They concentrate the majority of large-scale industrial and economic activities. This is particularly the case for the refinery and petrochemical industrial complexes in Mohammedia and the phosphates industries in Safi and Jorf Lasfar (Nakhli 2010). Seaside tourism is also booming with four tourist centres on the Atlantic coast: Agadir and its region, the pole El Jadida-Casablanca-Mohammedia, the pole Rabat-Témara-Skhira-Bouznika and Tangier-Tetouan (Nakhli 2010).

The composition and biogeographic value of plant communities associated with *L. mucronatum* along its distribution range are largely unexplored (Géhu and Biondi 1996). Similarly, the genome of this Moroccan endemic has not been the subject of any previous work. Therefore, in this study we (i) analysed the plant community composition and richness; (ii) estimated the genome sizes; and (iii) performed a cytogenetic characterization of *L. mucronatum* across North-South Atlantic Moroccan coastline.

Materials and methods

Study species, plant material and field sampling

Limonium mucronatum is a perennial plant (hemicryptophyte sometimes chamaephyte) endemic to Morocco (Fennane and Ibn Tattou 2005) (Figure 1). Flowering takes place in winter-spring (Sauvage and Vindt 1954; Fennane et al. 1999). Populations were sampled along the North-South Atlantic Moroccan coastline along a latitudinal (climatic) gradient from Rabat to Agadir (Figure 2) during the spring 2015 and tagged with Global Positioning System. Herbarium specimens for each studied site were deposited in the Herbarium Prof. João de Carvalho e Vasconcellos (LISI) and in the Herbarium of Scientific Institute of Rabat (RAB). Phytosociological relevés (Braun-Blanquet 1932) were carried out in the nine sites (one relevé per site) where *Limonium mucronatum* was found. Plants were identified using the keys from the Moroccan flora by Fennane et al. (1999, 2007, 2014). Each relevé was conducted on homogenous plots of 64 m^2 selected randomly where cover was estimated using the Braun-Blanquet scale (six classes – 1: isolated plants; 2: cover $<$ 5%; 3: 5–25%; 4:25–50%; 5: 50–75%; 6: $>$75%). The main characteristics of the sampled sites are shown in Table 1. The biogeographic origin of species was identified according to the available literature (Fennane and Ibn Tattou 1998; Carazo-Montijano and Fernández-López 2006). For each site, the proportion of different biogeographic elements was calculated, and the observed threats were identified.

For genome size estimations two to three leaves per individual were collected in four *L. mucronatum* populations (Table 2). The plant material was stored in labelled hermetic plastic bags and maintained at 4 °C until flow cytometric analyses. For cytogenetic analyses, seeds of mother plants were collected in the four abovementioned natural populations to establish *ex situ* plant collections. Seeds were germinated in a growth chamber (Rumed®) with a photoperiod of 18 h/6h of light and dark, respectively, and a temperature of 25 °C until germination (Róis et al. 2012). Seedlings were transferred to organic planting pots (*Jiffy*® pots) and maintained in similar growth conditions at the Instituto Superior de Agronomia (Lisbon, Portugal).

Plant community analysis

The plant community's composition of the nine sites with *L. mucronatum* was studied using ascending hierarchical classification (AHC) using the cover-abundance values of species. The relationships between the species richness of each of the biogeographic elements in the sites and the geographical (latitude, altitude) and climatic (precipitation, temperature) factors were studied using Spearman rank correlation. The differences between each of the biogeographic elements and among substrate types (rocky or sandy) were tested using Kruskal–Wallis non-parametric tests. Multivariate and univariate analyses were carried out using STATISTICA 10 (StatSoft Inc., Tulsa OK, USA).

Genome size and DNA ploidy levels estimations

Genome size and DNA ploidy levels were assessed using flow cytometry. Nuclei were isolated following the procedure of Galbraith et al. (1983): 0.5 cm^2 of fresh leaf tissue of *Limonium* was chopped with a razor blade, simultaneously with 0.5 cm^2 of fresh leaf tissue of the internal reference standard (*Pisum sativum* L. 'Ctirad'; 2C = 9.09 pg), in a Petri dish containing 1 mL of WPB buffer (Loureiro et al. 2007). The nuclear suspension was filtered using a 50 μm nylon mesh and 50 μg/mL of propidium iodide (Fluka, Switzerland) was added to stain the DNA. To avoid staining of double-stranded RNA, 50 μg/mL of RNAse (Fluka, Switzerland) was also added. After a 5 min incubation period, samples were analysed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Germany).

Individual estimates were obtained for 5–6 plants per population providing genome size estimates for each population. Additional plants were analysed using pooled sample strategy (5 individuals plus the reference standard analysed simultaneously) providing estimates of DNA ploidy only.

The value of genome size in mass units (2C in pg; *sensu* Greilhuber et al. 2005) was obtained using the following equation: *Limonium* sp. 2C nuclear DNA content (pg) = (*Limonium* sp. G_1 peak mean/reference standard G_1 peak mean) × genome size of the reference standard. Because the genome size value was obtained for several individuals that were also karyologically characterized (see below), it was possible to infer DNA ploidy levels for all the individuals analysed.

Descriptive statistics were calculated for genome size data (mean, standard deviation of the mean, coefficient of variation and minimum and maximum values). Statistical analyses were performed using SigmaPlot for Windows v. 12.5 (Systat Software, USA).

Chromosome counts and fluorescence in situ hybridization (FISH)

Chromosome counts were made to confirm the ploidy levels estimates based on the genome sizes obtained using flow

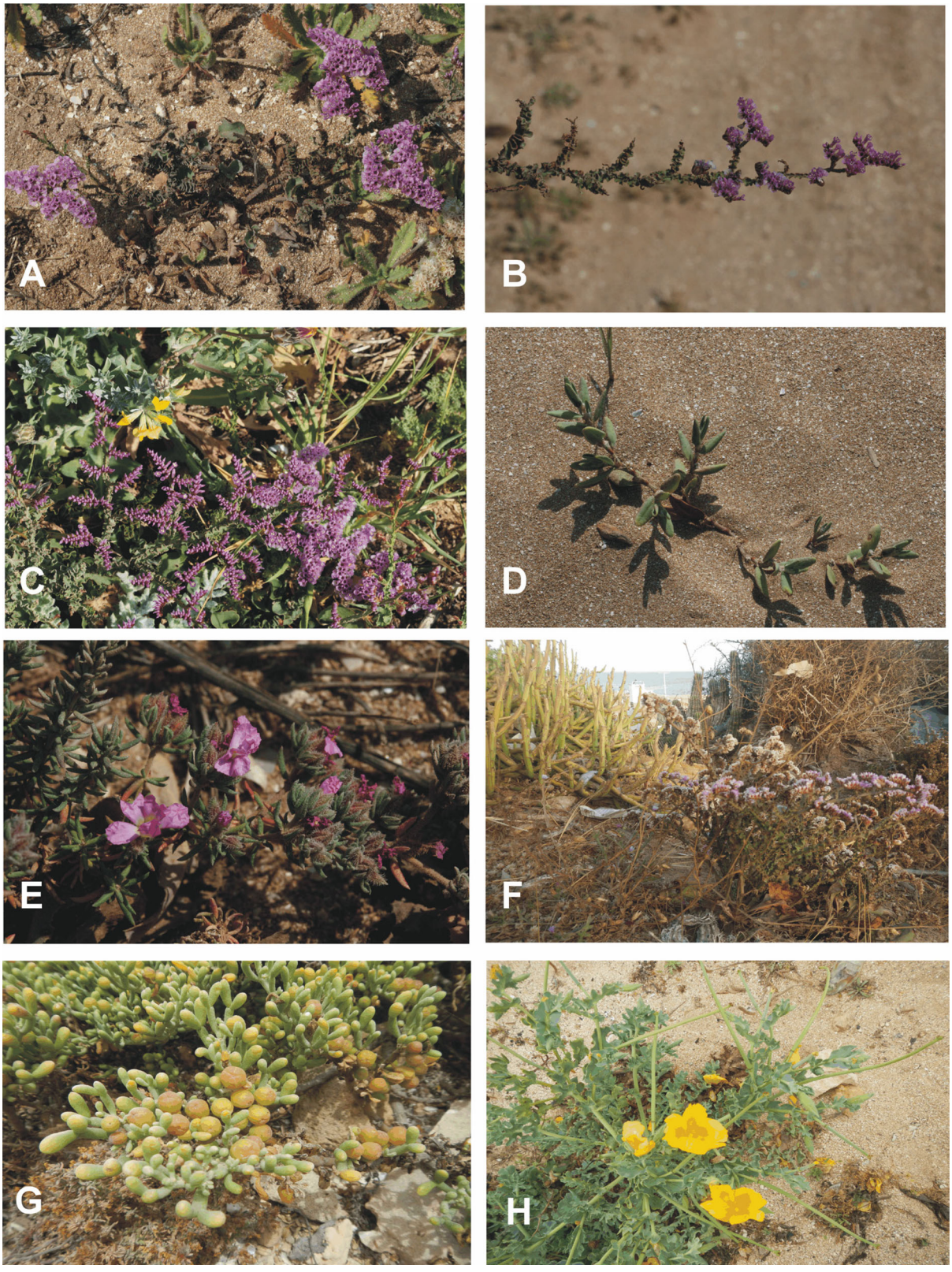

Figure 1. *Limonium mucronatum* and companion species. A, B: *Limonium mucronatum* (L.f.) Chaz.; C: *L. mucronatum* with *Lotus creticus* L.; D: *Polygonum maritimum* L.; E: *Frankenia laevis* L.; F: *L. mucronatum* with *Euphorbia regis-jubae* J.Gay and *Kleinia anteuphorbium* (L.) Haw.; G: *Zygophyllum fontanesii* Webb and Berthel.; H: *Glaucium corniculatum* (L.) Rudolph. Photographs by P. Grillas 2009 (A, B, C, D) and L. Rhazi 2015 (E, F, G, H).

cytometry. Cytological analyses of metaphase cells from eight *L. mucronatum* plants, two plants per population, were carried out on root tip cells. After collection, root tips were fixed in ethanol/acetic acid (3:1, v/v,) and prepared for chromosome squashes as in Caperta et al. (2008). Briefly, fixed roots were digested with an enzyme mixture cytohelicase, pectolyase, and pectinase at 3% each (Sigma-Aldrich, Germany). Fluorescence *in situ* hybridization (FISH) was

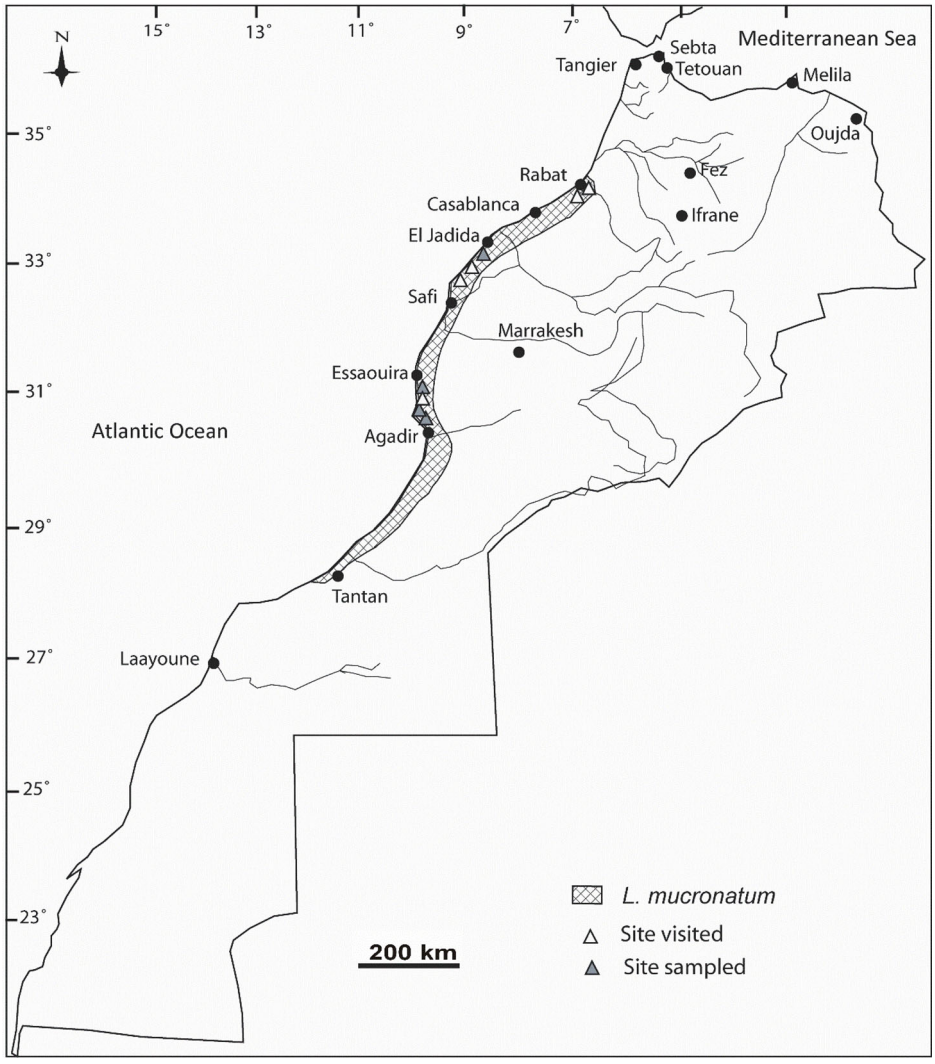

Figure 2. Distribution map of *Limonium mucronatum*. Visited and sampled sites are indicated in the figure legend.

Table 1. Main characteristics of the nine studied sites.

Site	Latitude (N)	Longitude (W)	Altitude (m)	Precipitation (mm)*	Temperature (°C)*	Bioclimate*	Substrate	Observed threat**
Rabat, Harhoura	34°00.821′	6°51.770′	5	523	17.9	Sub-humid	Sandy	Urbanization, facilities
Rabat, Sidi Abed	33°59.252	6°48.414′′	20	488	18.0	Sub-humid	Rocky	Urbanization, facilities
El Jadida, Sidi Bouzid	33°13.044′	8°33.951′	14	377	17.4	Semi-arid	Rocky	Urbanization, facilities
El Jadida, Jorf lasfar	33°05.204	8°39.362′	5	376	17.4	Semi-arid	Rocky	Chemical pollution (industrial)
El Jadida, Oualidia(s)	32°44.671′	9°01.619′	3	361	18.3	Semi-arid	Rocky	Agriculture, urbanization
Essaouira, Sidi Kaouki(s)	31°21.022′	9°47.804	4	279	17.5	Semi-arid	Sandy	Diffuse solid pollution
Essaouira, Imsouane(s)	30°50.818′	9°48.548′	135	293	16.7	Semi-arid	Sandy	Diffuse solid pollution
Agadir, Cap Ghir(s)	30°37.623′	9°52.694′	35	253	18.1	Semi-arid	Rocky	Diffuse solid pollution, facilities
Agadir, Taghazout	30°35.939′	9°46.516′	4	250	18.3	Semi-arid	Sandy	Urbanization

Climatic data (*) were obtained from Ionesco and Mathez (1966) (precipitation and temperatures correspond to mean annual values); Threats (**) identified in this study. (s) Sampled populations for flow cytometric and cytogenetic analyses.

Table 2. Genome size estimates, ploidy level and chromosome counts obtained in populations of the *Limonium mucronatum* sampled in Morocco (MA).

	Genome size (2C, pg)							
Limonium mucronatum population	Mean	SD	Min	Max	N	Chromosome counts	Ploidy level (N)	Voucher
El Jadida, Oualidia	5.28	0.06	5.20	5.38	6	12	2*x* (6)	LISI – 709/2015
Essaouira, Imsouane	5.18	0.05	5.12	5.25	5	12	2*x* (16)	LISI – 707/2015
Essaouira, Sidi Kaouki	5.23	0.06	5.16	5.33	5	12	2*x* (15)	LISI – 705/2015
Agadir, Cap Ghir	5.27	0.07	5.16	5.35	5	12	2*x* (8)	LISI – 699/2015

The following data are given for each population: mean, standard deviation of the mean (SD) and minimum (Min) and maximum (Max) values of the holoploid genome size (2C, pg), followed by sample size for genome size estimates (N); chromosome counts observed in this study and ploidy level (2*x*, diploid) followed by sample size in parenthesis are also provided. Vouchers of representative specimens are provided.

Table 3. List (in presence/absence) of species found in the nine sites with *Limonium mucronatum*.

	Code	Family	Status*	Harhoura	Sidi Abed	Sidi Bouzid	Jorf Lasfar	Oualidia	Sidi Kaouki	Imsouane	Cap Ghir	Tagha-zout
Ammophila arenaria (L.) Link	*Am.are*	Poaceae		1	.	.	.	.	.	.	.	.
Anacyclus radiatus Loisel.	*An.rad*	Asteraceae		1	1	1	1	.	.	.	.	.
Andryala mogadorensis Coss. ex Hook.f.	*An.mog*	Asteraceae	R?	.	.	.	.	.	1	.	1	.
Astydamia latifolia (L.f.) Kuntze	*As.lat*	Apiaceae	V	.	.	.	.	.	.	.	1	.
Atriplex ifniensis Caball.	*At.ifn*	Amaranthaceae		.	.	.	.	.	.	1	.	.
Bassia tomentosa (Lowe) Maire and Weiller	*Ba.tom*	Amaranthaceae	R?	.	.	.	.	.	.	1	1	1
Cakile maritima Scop.	*Ca.mar*	Brassicaceae		1	1	1	.	1	.	.	.	.
Calystegia soldanella (L.) R.Br. ex Schult.	*Ca.sol*	Convolvulaceae	RR	.	.	.	.	1	.	.	.	.
Crucianella maritima L.	*Cr.mar*	Rubiaceae		.	1	1	.	1	.	.	.	.
Cutandia maritima (L.) Benth.	*Cu.mar*	Poaceae		1	.	.	.	1	.	.	.	.
Elytrigia juncea (L.) Nevski	*El.jun*	Poaceae		.	.	.	1	.	1	1	.	.
Euphorbia paralias L.	*Eu.par*	Euphorbiaceae		1	.	1	1	1	.	.	.	1
Euphorbia regis-jubae J.Gay	*Eu.reg*	Euphorbiaceae		.	.	.	.	.	.	1	1	1
Frankenia laevis L.	*Fr.lae*	Frankeniaceae		1	1	1	.	1	.	.	.	.
Glaucium corniculatum (L.) Rudolph	*Gl.cor*	Papaveraceae		1	.	.	.	.	.	.	.	.
Kleinia anteuphorbium (L.) Haw.	*Kl.ant*	Asteraceae		.	.	.	.	.	.	.	1	1
Launaea arborescens (Batt.) Murb.	*La.arb*	Asteraceae		.	.	.	.	.	.	1	1	.
Limbarda crithmoides (L.) Dumort.	*Li.cri*	Asteraceae		.	.	1	.	.	.	.	.	.
Limonium densiflorum (Guss.) Kuntze	*Li.den*	Plumbaginaceae	R	.	.	.	1	.	.	.	.	.
Limonium mucronatum (L.f.) Chaz.	*Li.muc*	Plumbaginaceae		1	1	1	1	1	1	1	1	1
Limonium nydeggeri Erben	*Li.nyd*	Plumbaginaceae	RR	.	1	.	.	.	.	.	.	.
Limonium ovalifolium (Poir.) Kuntze	*Li.ova*	Plumbaginaceae	V	.	1	.	.	.	.	.	.	.
Limonium sinuatum (L.) Mill.	*Li.sin*	Plumbaginaceae		1	1	1	1	.	1	.	.	1
Lotus creticus L.	*Lo.cre*	Fabaceaee		1	1	1	1	.	.	.	.	.
Lycium intricatum Boiss.	*Ly.int*	Solanaceae		.	.	.	.	.	.	1	1	.
Medicago marina L.	*Me.mar*	Fabaceae		.	.	.	1	.	.	.	.	.
Mesembryanthemum crystallinum L.	*Me.cri*	Aizoaceae		.	.	.	.	.	1	.	.	.
Ononis tournefortii Coss.	*On.tou*	Fabaceae		.	.	.	.	.	.	1	1	1
Otanthus maritimus (L.) Hoffmanns. and Link	*Ot.mar*	Asteraceae		1	1	1	1	1	.	.	.	.
Paronychia argentea Lam.	*Pa.arg*	Caryophyllaceae		1	1	.	1	.	.	.	.	.
Plantago coronopus L.	*Pl.cor*	Plantaginaceae		1	1	1	.	.	.	.	.	.
Polycarpaea nivea (Aiton) Webb	*Po.niv*	Caryophyllaceae		.	.	.	1	.	1	1	1	1
Polygonum maritimum L.	*Po.mar*	Polygonaceae		1	.	.	1	1	.	.	.	1
Salsola kali L.	*Sa.kal*	Amaranthaceae		1	.	.	.	1	1	.	.	.
Sonchus tenerrimus L.	*So.ten*	Asteraceae		.	.	.	.	1	.	.	.	.
Spergularia purpurea (Pers.) G.Don	*Sp.pur*	Caryophyllaceae		1	.	.	.	.	.	.	.	.
Sporobolus pungens (Schreb.) Kunth	*Sp.pun*	Poaceae		1	1	.	.	.	.	.	.	.
Suaeda vera Forssk. ex J.F.Gmel.	*Su.ver*	Amaranthaceae		.	.	.	.	1	.	.	.	.
Zygophyllum fontanesii Webb and Berthel.	*Zy.fon*	Zygophyllaceae		.	.	.	1	1	.	.	.	.
Total			7	17	13	11	13	13	7	9	10	9

*The current status for Morocco (RR: very rare; R: rare; R? thought to be rare; V: vulnerable) as recorded in Fennane and Ibn Tattou (1998).

performed as previously described (Caperta et al. 2008). For rDNA labelling, two probes were used: (a) the pTa71 probe (45S rDNA, containing the *Triticum aestivum* 18S, 5.8S, 25S rDNA repetitive unit (Gerlach and Bedbrook 1979) which was labelled with biotin-dUTP (Roche®, Basel, Switzerland) using a nick translation kit (Roche®, Switzerland) and (b) the pTa794 containing a 420 bp long DNA sequence of wheat containing the 5S rRNA gene and the intergenic spacer (5S rDNA). This probe was generated from the clone pTa794 (Gerlach and Dyer 1980) and labelled with digoxigenin-11-dUTP (Roche, Switzerland) through PCR by using universal M13 "forward" (5′-CAG GGT TTT CCC AGT CAC GA-3′) and "reverse" (5′-CGG ATA ACA ATT TCA CAC AGG A-3′) sequencing primers. The thermal cycling programme was 94 °C for 1 min, 39 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 90 s, and 72 °C for 5 min.

For each slide 50 ng of pTa71 and 100 ng of pTa794 were used. These probes were diluted in a hybridization mixture composed by 50% formamide, 2× SSC (saline-sodium citrate buffer), 0.17% SDS (sodium dodecyl sulfate), 1 μg/μL salmon sperm, 10% dextran sulphate (stringency of 77%). Post-hybridization washes were done with a stringency of 85%, and bovine serum albumin 5% (w/v) was used as blocking reagent before probe detection with 2 μg g/mL of anti-digoxigenin fluorescein (Thermo Fisher Scientific, USA) and 5 μg g/mL of streptavidin-Cy3 (Sigma-Aldrich, Germany), as previously described (Caperta et al. 2008). After FISH, slides were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (1 mg/ml) in Vectashield antifade mounting medium (Vector Laboratories, USA). Samples were examined using a Zeiss Axioskop 2 epifluorescence microscope (Zeiss, Germany), and images were obtained using a Zeiss AxioCam digital camera (Zeiss, Germany) and digital images processed using Adobe Photoshop (Adobe Systems, USA).

Results and discussion

Limonium mucronatum communities

The phytosociological data revealed the occurrence of 39 species (Table 3), of which 87% are strictly associated with dunes and coastal cliffs (Atbib 1987; Géhu and Biondi 1996),

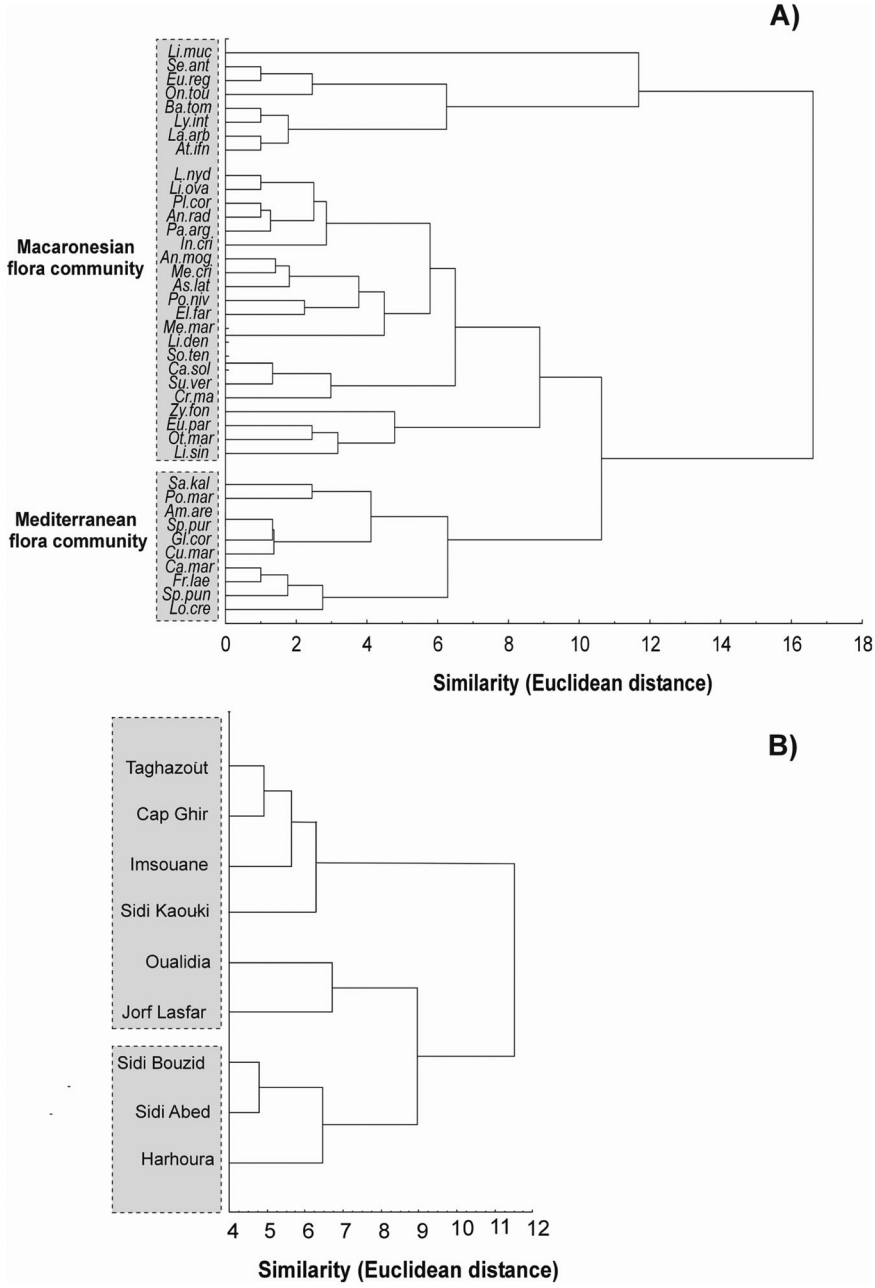

Figure 3. Dendrogram obtained by ascending hierarchical classification carried out on the phytosociological records of the nine sites studied (A: species; B: Sites). The meaning of the abbreviations of the species names is given in Table 2.

and 18% are rare for Morocco with high value for conservation (Table 3). These species belong to 18 families, of which the most represented are Asteraceae (18%), Plumbaginaceae (13%), Poaceae (10%) and Amaranthaceae (10%) (Table 3). The predominance of Asteraceae in *L. mucronatum* communities is similar to what was found in the total flora of Morocco, where this family totalizes *circa* 550 species (Fennane and Ibn Tattou 2012). On another hand, Plumbaginaceae that were strongly represented in the studied plant communities, are placed in the 18th rank in Morocco flora (Emberger and Maire 1941; Fennane and Ibn Tattou 2012).

Based on the results of the AHC (Figure 3A), two communities associated with *L. mucronatum* were distinguished (Figure 3A):

(1) *Mediterranean flora* community marked by the predominance of Mediterranean and Atlantic species (e.g., *Lotus creticus* L., *Ammophila arenaria* (L.) Link, *Frankenia laevis* L., *Cakile maritima* Scop.). This community was found on coastal sites with sub-humid bioclimate in the Rabat region (Harhoura, Sidi Abed) and in semi-arid bioclimate in El Jadida region (Jorf Lasfar, Oualidia and Sidi Bouzid) (Figure 3B). Although this community is dominated by Mediterranean and Atlantic species, it includes some Macaronesian elements (e.g., *Zygophyllum fontanesii* Webb and Berthel., *Polycarpaea nivea* (Aiton) Webb) in the north of Essaouira-Safi (Oualidia, Jorf Lasfar). However, the Macaronesian elements remain poorly represented compared to the predominant Mediterranean elements. These sites correspond to the northern limit of the Macaronesian flora, whose appearance in this region is due to

Table 4. Spearman rank correlations between total richness, richness in different biogeographic elements (Mediterranean and Atlantic; endemic; Macaronesian, subcosmopolitan; paleosubtropical) in the studied sites with geographic (latitude, altitude) and climatic (precipitation, temperature) factors.

Richness	Rho	*p*
Total		
Latitude	0.78	*
Altitude	−0.06	ns
Precipitation	0.81	**
Temperature	0.13	ns
Mediterranean and Atlantic		
Latitude	0.99	***
Altitude	−0.06	ns
Precipitation	0.97	***
Temperature	−0.23	ns
Endemic		
Latitude	−0.09	ns
Altitude	0.23	ns
Precipitation	−0.18	ns
Temperature	0.18	ns
Macaronesian		
Latitude	−0.91	***
Altitude	0.13	ns
Precipitation	−0.87	**
Temperature	0.19	ns
Subcosmopolitan		
Latitude	0.21	ns
Altitude	−0.55	ns
Precipitation	0.21	ns
Temperature	0.43	ns
Paleosubtropical		
Latitude	0.54	ns
Altitude	−0.09	ns
Precipitation	0.45	ns
Temperature	0.13	ns

Legend: ns = not significant, $^{*}p \leq 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the geomorphology of Safi (rocky cliffs) that promotes a favourable microclimate to these species (Géhu and Biondi 1998). These locations that are part of the sclerophyll forests ecoregion of Morocco (Benabid 2000), are well watered (Table 1) and subjected to frequent wet northern winds (Sauvage 1960). A similar plant community was found in the Atlantic littoral dunes in the north of Rabat (Atbib 1987) and in the coast of El Jadida-Safi (Géhu and Biondi 1996, 1998). From a phytosociological point of view, this Mediterranean-Atlantic flora community is connected to the association *Loto cretici-Ammophiletum arenariae* (Riv.-God. and Riv.-Mart. 1958) Riv.-Mart. 1964 em. Géhu and Sadki 1994. The major threats to the sites harbouring this plant community are urbanization and touristic facilities (Table 1), which are expanding on the coast of Morocco since the adoption of the "Plan Azur" in 2001 (Boumeaza et al. 2010; Nakhli 2010). The development of these facilities gradually reduces the area of occupancy of these communities by altering the quality of the habitat (pollution, physical disturbance).

(2) *Macaronesian flora* community characterized by a greater richness than in Moroccan-Canarian endemics (e.g., *Bassia tomentosa* (Lowe) Maire & Weiller, *Astydamia latifolia* (L.f.) Kuntze) and species typical from the Macaronesian-Morocco ecoregion (infra-Mediterranean stage), which are restricted to the southwest of the country (Quézel 1983; Benabid 2000). This community is characteristic of coastal sites with semi-arid bioclimates, slightly watered from the south of Oualidia to Agadir (Figure 3B). This community is characterized by a wealth of succulent elements (e.g., *Kleinia*

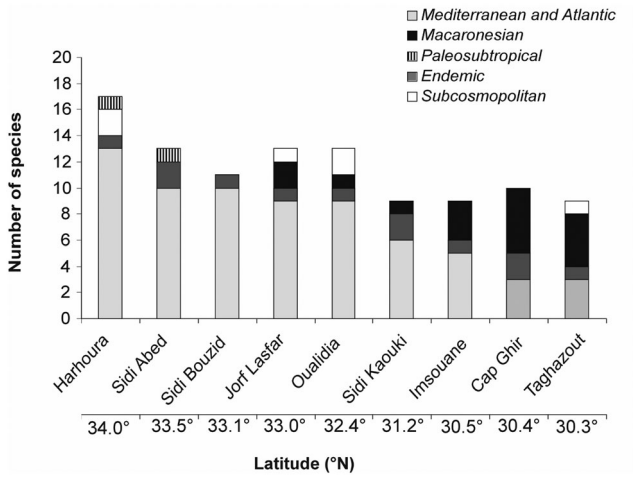

Figure 4. Proportion of the different biogeographic elements in *Limonium mucronatum* communities in the visited sites. These sites are ranked by decreasing latitude.

anteuphorbium (L.) Haw., *Euphorbia regis-jubae* J.Gay) in the south of Essaouira (Sidi Kaouki, Imsouane) and Agadir (Cap Ghir, Taghazout), where rainfall is low but atmospheric humidity is high due to Canary' influence (cold maritime current and the quasi-permanence of strong and cool northern winds). This region is known by an original floristic synendemic combination mainly associated with Canarian species and south Moroccan endemic species (Quézel 1983; Géhu and Biondi 1998; Benabid 2000). From a phytosociological point of view, this community can be associated with the *Limonio-mucronati-Astidamietum latifoliae* (Géhu and Biondi 1998) community. The most frequent anthropogenic pressures on the sites harbouring this community were urbanization, touristic facilities, pollution and agriculture (Table 1).

Species richness

The total species richness of *L. mucronatum* plant communities was positively correlated with latitude and precipitation; however, no significant correlation was found with altitude and temperature (Table 4). The northern sites with wet bioclimates (Harhoura, Sidi Abed) were significantly richer ($F = 6.47$; $df = 7$; $p = 0.0385$) than those under semi-arid bioclimate sites (Sidi Bouzid, Jorf Lasfar, Oualidia, Sidi Kaouki, Imsouane, Cap Ghir, Taghazout) (Figure 4). The richness of the communities in Mediterranean and Atlantic floristic elements was positively correlated with latitude and rainfall (Table 4), decreasing from north to south (Figure 4). Conversely, the richness of Macaronesian elements of the flora was negatively correlated with latitude and rainfall (Table 4). These Macaronesian elements were totally absent in the northern sites (Harhoura, Sidi Abed, Sidi Bouzid) and became predominant in the southern ones (Cap Ghir and Taghazout, Figure 4). The richness in other biogeographic elements (endemic, subcosmopolite, paleosubtropical) did not show any significant correlation with the geographical (latitude, altitude) and climatic factors (precipitations, temperatures) (Table 4). Moreover, the type of substrate (rocky or sandy) had no significant effect on the richness of *L.*

mucronatum plant communities (total, Mediterranean-Atlantic elements, Macaronesian, endemic, subcosmopolite, paleosubtropical elements) ($p > 0.05$). This suggests a dominant effect of the climate (quite contrasted along the latitudinal gradient) on the richness of the communities rather than the nature of the substratum. However, it is likely that soil chemistry (not measured in this study) contributed to the richness and composition observed in these communities.

Cytogenetic analysis

Flow cytometric analyses enabled us to unambiguously assess the ploidy level for 45 individuals and the genome size of 21 individuals from a total of four populations of *L. mucronatum* (Table 2; Figure 5). Generally, the quality of the flow cytometry histograms was very good, with the CV values of the G1 peaks of the sample and standard averaging 4.21% and 2.78%, respectively. A single cytotype was detected ($2x = 5.24$ pg/2C). The genome size value obtained is a new addition. In comparison with other *Limonium* species, this value is closer to that found in triploid *Limonium algarvense* Erben and *Limonium normannicum* Ingr., with an Atlantic Europe and Moroccan distribution (Caperta et al. 2018). No significant differences in genome size were observed among individuals ($p < 0.001$), which were ploidy homogeneous (Figure 5; Table 2). Constancy in ploidy was also observed in natural populations of other euploid and polyploid *Limonium* complexes in their entire distribution range such as complexes *Limonium binervosum* (G.E.Sm.) C.E.Salmon, *Limonium ovalifolium* (Poir.) Kuntze, and *Limonium vulgare* Mill. (Caperta et al. 2017; Róis et al. 2018).

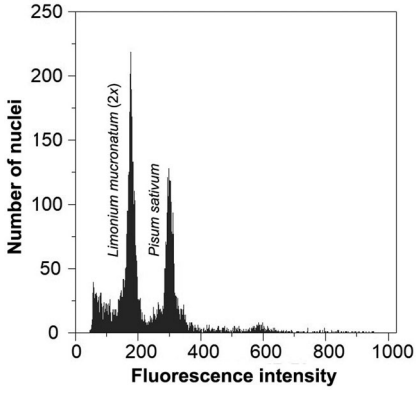

Figure 5. Flow cytometric analyses made in *Limonium mucronatum*. Flow cytometric histograms of propidium iodide stained nuclei of *L. mucronatum* analysed simultaneously with the internal standard *Pisum sativum* L. 'Ctirad'.

Karyotype structure of all studied accessions showed a diploid karyotype with $2n = 12$ chromosomes as previously recorded (Fedorov 1974), with well-defined secondary constrictions in chromosome 2 pair (Figure 6). A large metacentric pair (4–5 μm), a small metacentric pair (1.5–2.5 μm), and four pairs of submetacentric (2–4 μm) were visible. FISH analysis revealed chromosome localization of 45S and 5S rDNA sites in a chromosome pair in the distal region of the long arm of a submetacentric chromosome (chromosome 2) (Figure 6). 5S rDNA sites were observed in the proximal region of the long arm in a pair of submetacentric chromosomes (chromosome 3) (Figure 6).

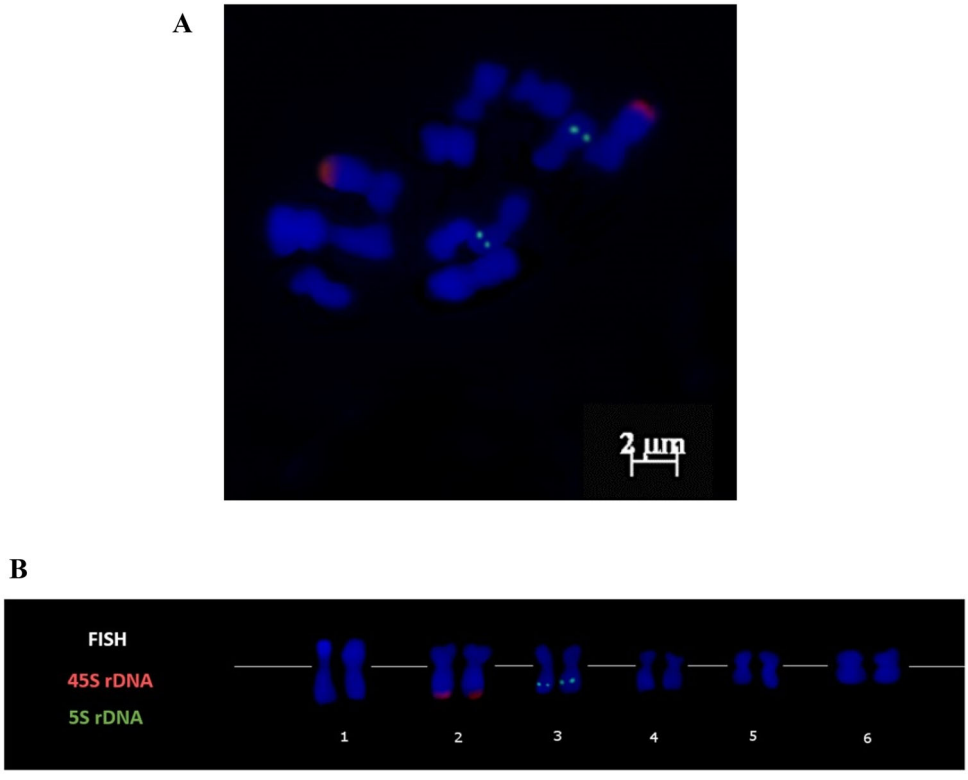

Figure 6. Chromosome spreads of *Limonium mucronatum* after fluorescence *in situ* hybridization (FISH). A. Merged fluorescent images after FISH with 45S rDNA (red) and 5S rDNA (green), and DAPI stained chromosomes (blue). B. Karyotypes of metaphase plates of *L. mucronatum* after FISH with 45S rDNA (red) and 5S rDNA (green).

Different cytogenetic studies revealed a remarkable variability of chromosome polymorphisms in *Limonium* such as diploid ($2n = 2x = 12$, 14, 15, 16, 17, 18), triploid ($2n = 3x = 24$, 25, 26, 27, 28), tetraploid ($2n = 4x = 32$, 33, 34, 35, 36), pentaploid ($2n = 5x = 43$), and hexaploid ($2n = 6x = 51$, 54, 56) species (Fedorov 1974; Erben 1978, 1979, 1993; Goldblatt 1981; Goldblatt and Johnson 2006; Castro and Rosselló 2007; Róis et al. 2012, 2018; Cortinhas et al. 2015; Caperta et al. 2017). Whereas northwest Atlantic and Mediterranean representative species typically present basic chromosome numbers $x = 8$ and $x = 9$ in euploid or multiple combinations originating polyploids (Erben 1978), in Macaronesian *Limonium* taxa. Three basic chromosome numbers of $x = 6$, 7, 8 are usually found (Febles and Pérez-Rodríguez 2004; Lledó et al. 2011). As other species included in *Limonium* sect. *Ctenostachys* represented in Morocco, Canary Islands and Cape Verde, *L. mucronatum* appears to have distinct evolutionary origins of Mediterranean *Limonium*.

Conclusions and plant conservation remarks

First, the study conducted on *L. mucronatum* along the Rabat-Agadir littoral highlights a high degree of originality of the plant communities associated with this species, with numerous rare and endemic species (Morocco, Canary Islands), with high conservation value. These communities are very rich in Mediterranean and Mediterranean-Atlantic elements towards the north (from El Jadida: Sidi Bouzid); while towards the south, Macaronesian elements predominate. This change in communities' composition from Rabat to the south of Agadir is related to the bioclimatic factors (subhumid to the north and semi-arid to the south), well-marked along a latitudinal gradient. Although *L. mucronatum* plant communities are very original, they are subject to many anthropogenic pressures that can lead to their impoverishment or local destruction.

Second, because cytogenetic analyses in natural populations showed constancy in ploidy levels and no chromosome polymorphisms throughout the studied geographic range, having diploid individuals with six chromosome pairs, these data will facilitate future management in situ of natural populations of this species. Thus, these results are fundamental in order to delineate better conservation strategies to preserve this rare and endemic diploid species under strong anthropogenic pressure.

At least for *L. mucronatum* plant communities with Macaronesian elements, the location of the entire coastal strip in the south of Essaouira, within the Arganeraie biosphere reserve (Argan trees, *Argania spinosa* (L.) Skeels) could promote the effective conservation of these plant communities constituting a true "floristic jewel" of the Moroccan Atlantic coast.

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No potential conflict of interest was reported by the authors.

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