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Taxonomic complexity in the halophyte *Limonium vulgare* and related taxa (Plumbaginaceae): insights from analysis of morphological, reproductive and karyological data

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• **Background and Aims** *Limonium* is a well-known example of a group of plants that is taxonomically complex due to certain biological characteristics that hamper species' delineation. The closely related polyploid species *Limonium vulgare* Mill., *L. humile* Mill. and *L. narbonense* Mill. are defined species and can be used for studying patterns of morphological and reproductive variation. The first two taxa are usually found in Atlantic Europe and the third in the Mediterranean region, but a number of intermediate morphological forms may be present alongside typical examples of these species. This study attempts to elucidate morphological, floral and karyological diversity representative of these taxa in the Iberian Peninsula.

• **Methods** The extent of morphological differentiation was tested through comparison of 197 specimens from both Portugal and Spain using 17 descriptive morphological characters and 19 diagnostic morphometric characters. Analyses of floral morphisms (heterostyly and pollen–stigma dimorphism) and karyological determinations were also conducted.

• **Key Results and Conclusions** Discriminant analysis using morphometric variables reliably assigned individuals in natural populations to their respective groups. In addition, the results provide the first direct evidence that *L. narbonense* and a new species, ***Limonium maritimum*** Caperta, Cortinhas, Paes, Guara, Espírito-Santo and Erben, **sp. nov.**, related to *L. vulgare* are present on Portuguese coasts. Most of these species are found together in mixed populations, especially *L. vulgare* and *L. narbonense*. It is hypothesized that taxonomic biodiversity found in sites where distinct species co-occur facilitates the evolutionary processes of hybridization, introgression and apomixis. This study therefore contributes to the elucidation of the taxonomic diversity in *L. vulgare*-related species and may also help in implementing future conservation programmes to maintain the evolutionary processes generating biodiversity.

Key words: Halophyte, heterostyly, karyological polymorphisms, *Limonium humile*, *L. maritimum*, *L. narbonense*, *L. vulgare*, morphometry, pollen–stigma dimorphism, polyploidy, Plumbaginaceae, taxonomy.

INTRODUCTION

Taxonomically complex groups (TCGs) such as the halophytic *Limonium* spp. (*Plumbaginaceae*), typically found in coastal areas and saline steppes (Erben, 1993; Kubitzki, 1993; van der Maarel and van der Maarel-Versluys, 1996), harbour significant biodiversity. In some of these TCGs, some form of uniparental reproduction (e.g. selfing, apomixis or gynogenesis) is usually present, and hybridization occurs to some degree among its members, whose biological diversity defies simple classification into discrete species (Palacios *et al.*, 2000; Lledó *et al.*, 2005). Therefore, conserving biodiversity within these TCGs is essential because diversity is generated and maintained by facilitating the evolutionary interactions among their members (i.e. hybridization and introgression), rather than by preserving the taxonomic entities that such evolution produces (Ennos *et al.*, 2005). However, due to the biological characteristics they present, there are difficulties in delimitating taxa. Therefore, clarifying taxonomic biodiversity of well-represented taxa from the European coasts but where taxonomic uncertainties currently exist is crucial for their conservation.

In the TCG *Limonium*, notably high levels of karyological polymorphisms linked with polyploidy (spanning triploids to octoploids) and aneuploidy are found (Dolcher and Pignatti, 1971; Erben 1978, 1979; Dawson, 1990; Arrigoni and Diana, 1993; Georgakopoulou *et al.*, 2006; Castro and Rosselló, 2007; Róis *et al.*, 2012). Triploid species seem to be highly concentrated in the western Mediterranean region, while tetraploid species and taxa with higher ploidy levels occur mainly in the Atlantic coasts and in the eastern Mediterranean region (Cowan, 1998). Moreover, descriptive morphological (e.g. Pignatti, 1971, 1972), morphometric and sequence data from both plastid and nuclear DNA (Lledó *et al*., 1998, 2001, 2005; Palacios *et al.*, 2000) have been used to elucidate the enormous taxonomic complexity of this genus. The closely related polyploid saltmarsh species, *L. vulgare* Mill. (the type of the genus; known as *Statice limonium* L.; *Statice*, *nom. rej*. vs. *Armeria*; Greuter *et al.,* 2000), *L. humile* Mill. and *L. narbonense* Mill., have long been recognized as distinct species (Erben, 1993). *Limonium vulgare* is known so far as a tetraploid ($2n = 4x = 36$) and *L. humile* as a hexaploid ($2n = 6x = 54$) (Dawson, 1990); on the other hand, the *L. narbonense* species complex comprises four species with diverse ploidy levels, namely *L. hirsuticalyx* ($2n = 2x = 18$), *L. narbonense* ($2n = 4x = 36$) (Erben, 1979; Artelari, 1992; Georgakopoulou *et al.*, 2006), *L. breviopetalum* ($2n = 6x = 54$) and *L. pagasaeum* ($2n = 8x = 72$) (S. Brullo and M. Erben, unpubl. data). *Limonium vulgare* and *L. humile* occur in Atlantic Europe (Boorman, 1967, 1971; Erben, 1993; Dawson and Ingrouille, 1995), whereas *L. narbonense* has a patchy distribution in the Mediterranean coastal region (Erben, 1993; Crespo and Lledó, 1998; Pandža *et al.*, 2007).

The presence of salt glands in plants from these species allows them to remove excess salts in the soil water (Flowers and Colmer, 2008; Grigore *et al.*, 2014). For example, in *L. vulgare* and *L. narbonense*, salt glands are below the surface in leaves and sepals, and at the surface of the petals (Ana R. Pina, Generosa Teixeira and Ana D. Caperta, unpubl. data). Fascinating reproductive systems associated with floral polymorphisms such as heterostyly and pollen–stigma dimorphisms are also found in species of *Limonium* (Baker 1948, 1953*a*, 1966). Of these, heterostyly is a morphological and reproductive polymorphism in which plant populations are composed of two (distyly) or three (tristyly) floral morphs that differ reciprocally in the height of their sytles and anthers, thus preventing self- and intramorph fertilization (Ganders, 1979). Also, pollen and stigma dimorphisms in which two pollen grain types (A- and B-pollen) that differ in their exine surface patterns and germinate on distinct stigma types (*cob*-like and papillose) are frequent (Baker, 1948, 1953*a*, 1966; Nowicke and Skvarla, 1977). Both flower heteromorphies are linked with a sporophytic self-incompatibility system preventing self-fertilization. Thus, in monomorphic populations, self-incompatible plants can only produce seeds through apomixis (asexual reproduction via seeds) (Baker, 1966; Richards, 1997). For example, in *L. vulgare*, meiotic (sexual) embryo sacs are formed (D'Amato, 1940) while in triploid ($2n = 3x = 27$) *Statice oleaefolia* var. *confusa*, meiotic embryo sacs together with apomictic (non-meiotic) embryo sacs are produced (D'Amato, 1949).

Due to the geographical location of Portugal, at the confluence between the Atlantic Ocean and the Mediterranean Sea, wide coastal areas provide an exceptional opportunity to study *L. vulgare* and related taxa that, according to the findings of previous authors, do not share distribution areas (Baker, 1953*a*, 1966; Erben, 1993; Dawson and Ingrouille, 1995). Both *L. vulgare* and *L. humile* are recognized in saltmarshes in Portugal (Erben, 1978; Franco, 1984; Costa *et al.*, 2012). Furthermore, earlier morphological studies by some of the authors (A.D.C. and M.E., unpublished data) showed that these species present variants in the coasts of Portugal. However, knowledge of the geographic distribution, morphological variation, ploidy levels and reproductive characters is lacking. In this study, detailed biometric surveys, floral morphisms and karyological analysis revealed, for the first time, that mixed populations of *L. vulgare* and related taxa occur along the Portuguese coasts.

MATERIALS AND METHODS

Study species

Limonium vulgare is a perennial rossulate chamaephyte with short spikes with densely arranged spikelets (Salmon, 1905*a*; Erben, 1993). In contrast to *L. vulgare*, *L. humile* is more loosely branched with long spikes and sparse spikelets (Salmon, 1905*b*; Erben, 1993), and *L. narbonense* forms taller plants with wider leaves and a longer scape than *L. vulgare* (Erben, 1993). These latter two species are putative obligate outcrossers (Baker, 1948, 1953*a*, *b*; Erben, 1979; Georgakopolou *et al.*, 2006), while *L. humile* is a probable facultative inbreeder (Boorman, 1968; Dawson and Ingrouille, 1995).

Study area, plant sampling and growth conditions

Field surveys were carried out along Portuguese continental coasts during July to September in 2010 and 2011. Specimens were collected in populations located in Tejo (T), Sado (S) and Mira (M) estuaries, in the coastal lagoons of Aveiro (Ria de Aveiro, A) and Ria Formosa (F), and in Veiga beach (Viana do Castelo, V). As populations were variable in size, different numbers of plants were collected at different sites (details in Table 1). All populations were tagged with a Global Positioning System and mapped using ArcGIS Desktop 10.0 (ESRI).

A total of 108 plants were sampled from 19 sites, then pressed, dried, labelled correspondingly and deposited in João de Carvalho e Vasconcellos Herbarium, Instituto Superior de Agronomia – LISI (abbreviation according to Holmgren,

TABLE 1. *Site locations surveyed for* Limonium vulgare *and related taxa*

Population	Site location/Province	Geographic co-ordinates	*n*
Viana (V)	Praia da Veiga, Areosa/Minho	41·72836/−8·87155	9
Aveiro (A)	Gafanha do Carmo-Encarnação/ Beira Litoral	40·62213/−8·73697	15
	Cais da Bestida, Torreira/Beira Litoral	40·7599/−8·67680	2
	Tijosa, Ovar/Beira Litoral	40·82073/−8·6497	2
	Ribeira da Aldeia, Estarreja/Beira Litoral	40·80145/−8·63625	5
	Quintas do Norte/Beira Litoral	40·79529/−8·67325	2
	Boco/Beira Litoral	40·58948/−8·68797	9
Tejo (T)	Sítio das Hortas, Alcochete/ Estremadura	38·76044/−8·93741	7
	Entroncamento, Alcochete/ Estremadura	38·74879/−8·92208	2
	Amora/Estremadura	38·62670/−9·11028	3
Sado (S)	Carrasqueira/Estremadura	38·41218/−8·75293	4
	Mouriscas/Estremadura	38·52844/−8·80435	8
	Tróia/Estremadura	38·46995/−8·86770	9
	Gâmbia/Estremadura	38·54851/−8·75816	2
	Salinas de Monte Novo/ Estremadura	38·44548/−8·70153	2
Mira (M)	Vila Nova de Mil Fontes/Alentejo	37·72775/−8·77093	6
	Casa Branca/Alentejo	37·66634/−8·72009	12
	Moinho da Asneira/Alentejo	37·73069/−8·75450	4
Formosa (F)	Pedras D'el Rei/Algarve	37·08613/−7·66277	5

Specimens were sampled in the provinces of Minho (North), Beira Litoral (West), Estremadura (West), Alentejo (Southwest) and Algarve (South).

n, number of individuals sampled.

TABLE 2. *Morphological descriptive and morphometric characters used for discrimination of* Limonium vulgare-*related taxa and collected individuals*

Morphological character* (Code)	(1) Present	(0) Absent	Units
Apex of inner bract centre (AIBC)	Yes	No apex	
Calyx and inner bract size relation (CIBSR)	Calyx > inner bract	Calyx < inner bract	
Calyx with trichomes (CT)	Yes	No	
Extension of the apex of inner bract centre (EAIBC)	Toward the margin	Below the margin	
First order sterile branches (SB1)	Yes	No	
Inflorescence type (IT)	G type*	Other	
Inner bract centre (IBC)	Membranaceous	Fleshy	
Leaf apex (LA)	yes	No	
Leaf shape (LS)	Oblongo-lanceolate	Other	
Margin of inner bract (MIB)	Hyaline	Non-hyaline	
Margin of inner bract centre (MIBC)	Dentate	Non-dentate	
Margin of middle bract (MMB)	Hyaline	Non-hyaline	
Margin of outer bract (MOB)	Hyaline	Non-hyaline	
Presence of salt (PS)	Yes	No	
Regularity of inner bract centre (RIBC)	Irregular	Regular	
Second order sterile branches (SB2)	Yes	No	
Venation (Vs)	Peninerved	Other	
Lamina length (LL)			cm
Maximum calyx length (MCL)			mm
Maximum distance of spikelets (MDS)			mm
Maximum inner bract length (MIBL)			mm
Maximum inner bract width (MIBW)			mm
Maximum limb/tube ratio (MLTR)			NA
Maximum lamina width (MLW)			cm
Maximum middle bract length (MMBL)			mm
Maximum middle bract width (MMBW)			mm
Maximum number of florets per spikelet (MNFS)			NA
Maximum number of spikelets per cm (MNSC)			NA
Maximum outer bract length (MOBL)			mm
Maximum outer bract width (MOBW)			mm
Maximum spike length (MSL)			cm
Petal length (PL)			mm
Scape first ramification angle (SFRA)			sin
Scape height (SH)			cm
Scape height to the first ramification (SHFR)			cm
Stalk length (SL)			cm

*Acronyms according to Erben (1993), Dawson and Ingrouille (1995) and Róis *et al.* (2013).
NA, not applicable.

TABLE 3. *Number and percentage of qualitative characters based on their presence in reference species* Limonium vulgare*,* L. humile *and* L. narbonense *and in collected individuals from natural populations*

Character	*L. humile* ($n = 13$)	*L. narbonense* ($n = 18$)	*L. vulgare* ($n = 16$)	Aveiro ($n = 52$)	Mira ($n = 22$)	Ria Formosa ($n = 17$)	Sado ($n = 30$)	Tejo ($n = 20$)	Viana ($n = 9$)
IT	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	29 (96·67)	20 (100)	9 (100)
Vs	11 (84·62)	18 (100)	16 (100)	52 (100)	22 (100)	17 (100)	29 (96·67)	20 (100)	9 (100)
LS	11 (84·62)	18 (100)	16 (100)	52 (100)	22 (100)	17 (100)	29 (96·67)	20 (100)	5 (55·56)
LA	8 (61·54)	17 (94·44)	15 (93·75)	50 (96·15)	20 (90·91)	16 (94·12)	10 (33·33)	16 (80)	7 (77·78)
PS	10 (76·92)	18 (100)	16 (100)	47 (90·38)	22 (100)	12 (70·59)	21 (70)	12 (60)	7 (77·78)
SB1	5 (38·46)	4 (22·22)	6 (37·5)	25 (48·08)	17 (77·27)	4 (23·53)	11 (36·67)	11 (55)	5 (55·56)
SB2	8 (61·54)	10 (55·56)	10 (62·5)	34 (65·38)	20 (90·91)	6 (35·29)	24 (80)	19 (95)	8 (88·89)
MOB	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
MMB	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
MIB	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
MIBC	12 (92·31)	15 (83·33)	4 (25)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
RIBC	12 (92·31)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
IBC	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
AIBC	10 (76·92)	10 (55·56)	13 (81·25)	41 (78·85)	16 (72·73)	14 (82·35)	17 (56·67)	8 (40)	9 (100)
EAIBC	0 (0)	1 (5·56)	0 (0)	2 (3·85)	0 (0)	1 (5·88)	0 (0)	0 (0)	4 (44·44)
CIBSR	13 (100)	18 (100)	16 (100)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)
CT	13 (100)	5 (27·78)	15 (93·75)	52 (100)	21 (95·45)	17 (100)	30 (100)	20 (100)	9 (100)

Details of acronyms are given in Table 2.
n, number of individuals.

1990). In some populations, seeds were collected for establishing experimental collections.

Descriptive morphological and morphometric analysis

Descriptive morphological (qualitative) and morphometric (quantitative) data were used in this study in both specimens collected in natural populations and reference specimens from herbaria collections. Reference specimens identified as *L. vulgare* ($n = 17$), *L. narbonense* ($n = 17$) or *L. humile* ($n = 13$), as well as specimens collected in natural populations ($n = 108$) deposited in herbaria of Spain and Portugal, namely Herbário da Universidade do Porto – PO, Herbário da Universidade de Coimbra – COI, Herbário do Museu Nacional de História Natural – LISU, Herbário João de Carvalho e Vasconcellos do Instituto Superior de Agronomia – LISI, Herbário da Estação Agronómica Nacional – LISE, Herbario del Real Jardín Botánico de Madrid – MA, Herbario del Jardín Botánico de la Universidad de Valencia – VAL and Herbario de la Sociedad de Ciencias Aranzadi – ARAN, were used (abbreviations according to Holmgren, 1990) (see Appendices 1 and 2).

For each specimen, 17 descriptive morphological characters were scored and 19 quantitative characters measured (Table 2) as previously reported for other *Limonium* spp. (Erben, 1978, 1993; Ingrouille, 1984; Ingrouille and Stace, 1986; Dawson and Ingrouille, 1995). For descriptive characters, a matrix was generated using the observed data, and matrix elements corresponded to the number of individuals that, in a given population, present a particular character. Using this matrix, correspondence analysis (CA) (Foucart, 1982; Podani, 1994) using NTSYS-PC v. 2.21 software (Rohlf, 2009) was conducted. To confirm populations with characters in equivalent proportions, χ^2 tests of independence were carried out (Sokal and Rohlf, 2012).

The morphometric variables were first tested for deviations from a normal distribution using a Kolmogorov–Smirnov test (Zar, 2010; Sokal and Rohlf, 2012). Non-parametric Kruskal–Wallis analyses of variance were also used (Zar, 2010; Sokal and Rohlf, 2012). To test possible affinities between the sampled specimens, standarized principal component analysis (PCA) was carried out on a matrix with the 197 cases corresponding to reference specimens, specimens from distinct beach/estuaries/lagoons and the quantitative characters (morphometric variables) using the NTSYS-PC v. 2.21 software (Rohlf, 2009). Complementary, canonical discriminant analysis (CDA) was conducted with SPSS 20 for Windows on the same matrix of morphometric data.

Determinations of floral morphs and seed germination analysis

A total of 97 dried specimens were used for floral morph determinations. Briefly, herbarium materials were first re-hydrated in distilled water for 15 min. Flower morph determinations were carried out by observing differences in the lengths of the pistil and stamens (heterostyly) as well as stigma and pollen types, as previously described (Baker, 1948; Erben, 1978). The dissected organs were covered with water or with a drop of basic

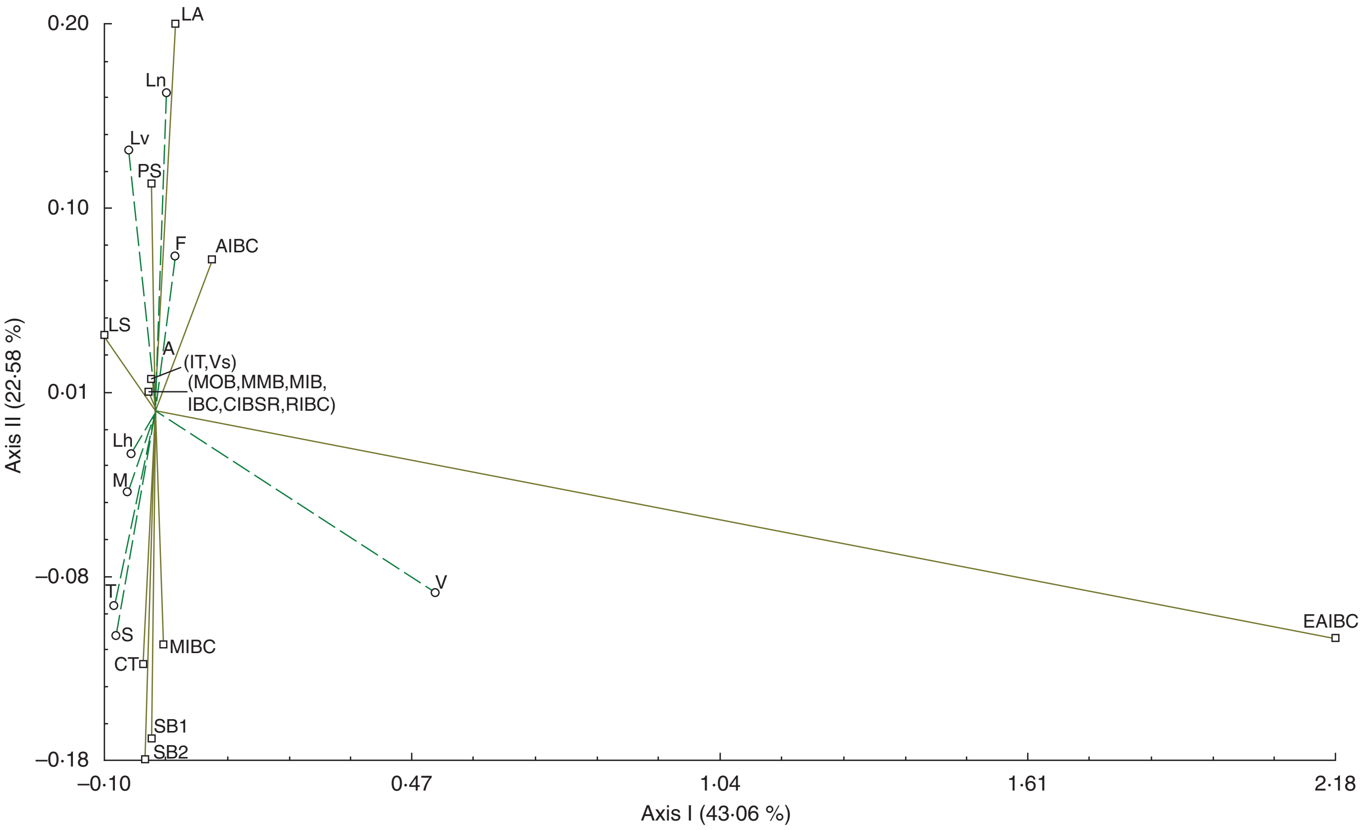

FIG. 1. The first two axes of correspondence analysis based on 17 qualitative characters. Percentages of total variance explained by the functions are given in parentheses; details of acronyms are given in Table 2.

fuchsin (0·05 %)–hydroalcoholic glycerin solution as described in Suárez-García *et al.* (2009). Preparations of pollen and stigmas were observed using an optical microscope (Leitz hm-lux 3). Statistical analysis of data was performed through CA (Foucart, 1982; Podani, 1994) using NTSYS-PC v. 2.21 software (Rohlf, 2009). χ^2 tests of independence were used (Zar, 2010; Sokal and Rohlf, 2012) to test similarities among populations in relation to the typologies analysed.

Estimations of seed set and germination tests were performed as described in Róis *et al*. (2012).

Karyotyping

Seven plants from experimental collections established using seeds from Aveiro (A) and Tejo (T) populations were used for karyotyping. Root tips were excised and then treated with a 2 mM 8-hydroxyquinoline solution for 2·5 h at 4 °C in the dark and subsequently for 2·5 h at room temperature to induce metaphases, as described in Róis *et al*. (2012). Then, root tips were fixed in 3:1 (v/v) absolute ethanol/glacial acetic acid fresh solution overnight and stored at −20 °C until used. Next, root tips were digested with a 2 % cellulase (Sigma), 2 % cellulase 'Onozuka R-10' (Serva) and 2 % pectinase (Sigma) enzyme solution in Enzyme Buffer (40 mL of 0·1 M citric acid-1-hydrate and 60 mL of 0·1 M sodium citrate dihydrate; pH 4·8) for 3 h at 37 °C, as described in Caperta *et al*. (2008). Squashes were made in 60 % acetic acid, and preparations were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (1 mg mL^{-1}) diluted in Citifluor antifadent mounting solutions (Agar Scientific). Cell preparations were observed using a Zeiss Axioskop 2 fluorescence microscope. Images were collected with an AxioCam MRc5 digital camera (Zeiss) and further processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA, USA). Chromosome counts were made on microphotographs of mitotic metaphase spreads with the same degree of chromosomal condensation in at least three individuals from each population.

RESULTS

Morphometric rather than descriptive morphological characters discriminate among species populations

Analysis of morphological data revealed that the characters CIBSR, IBC, IT, LS, MIB, MMB, MOB, PS, RIBC and Vs (see Table 2 for a list of characters and their acronyms) were practically coincident in all populations (>60 %), whereas AIBC, CT, EAIBC, LA, MIBC, SB1 and SB2 were less coincident (<50 %) (Table 3). Corrsepondence analysis of these variables

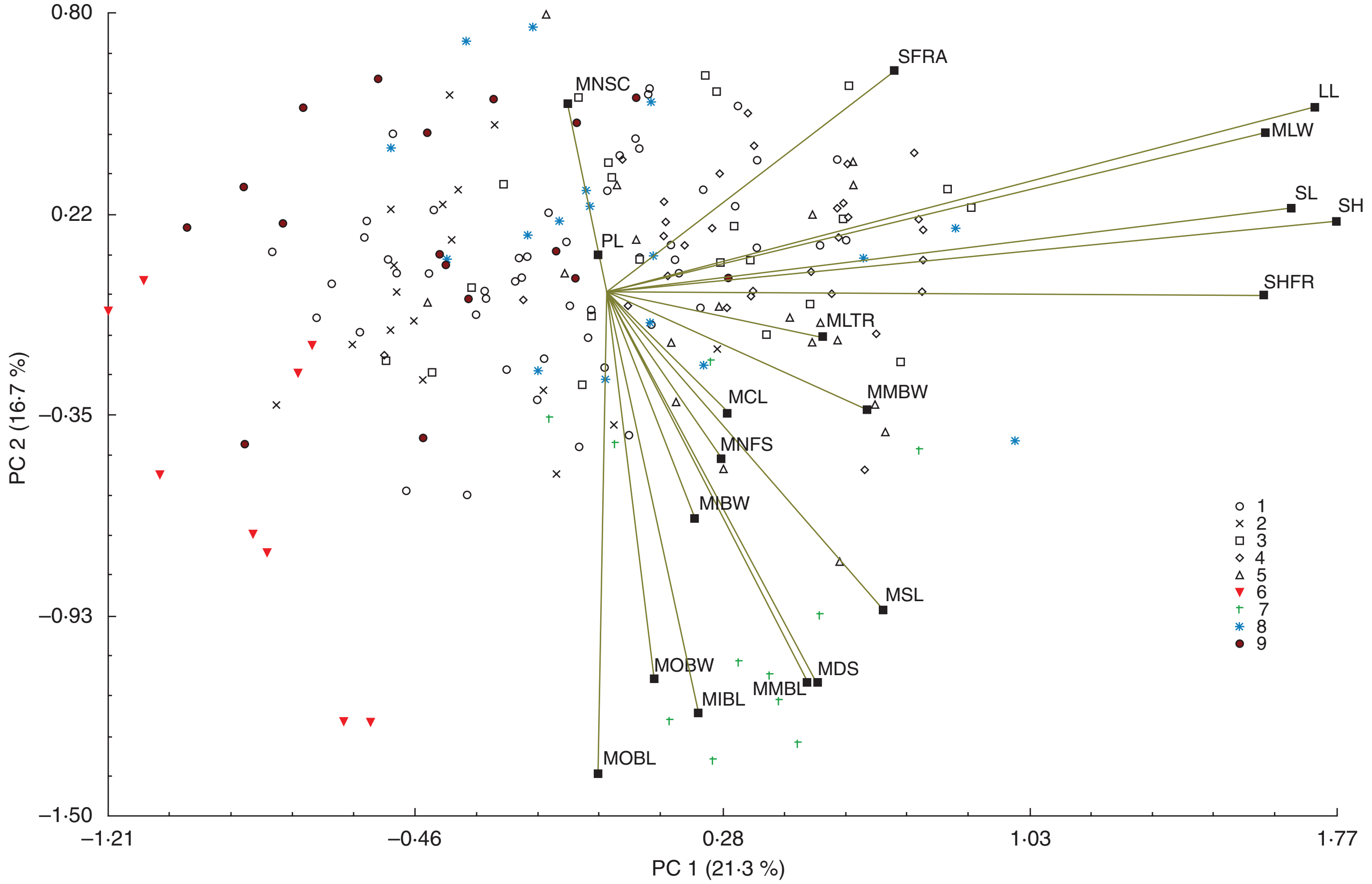

FIG. 2. The first two axes of principal component analysis based on 19 morphometric characters. Percentages of total variance explained by the functions are given in parentheses; details of acronyms are given in Table 2. 1: Aveiro; 2: Mira; 3: Sado; 4: Tejo; 5: Formosa; 6: Praia da Veiga, Viana do Castelo; 7: *L. humile*; 8: *L. narbonense*; 9: *L. vulgare*.

accounted for 65·64 % of the variation in the first two dimensions (axes), and 56·83 % in the first and third dimensions (axes) (Fig. 1). In most populations, the specimens studied exhibited equivalent proportions of the characters analysed ($\chi^2 = 97{\cdot}379$; $P = 0{\cdot}9798$). An exception to this was the V population which was significantly ($\chi^2 = 39{\cdot}814$; $P < 0{\cdot}001$) different from the reference specimens *L. narbonense* and *L. vulgare* and other populations. The first dimension also separated EAIBC in relation to all other variables ($\chi^2 = 38{\cdot}951$; $P < 0{\cdot}001$), infrequent in most specimens, but present in all V individuals.

Among morphometric variables, only two fitted a normal distribution, SH and SHFR, whereas the other nine failed to do so, even after a logarithmic transformation. Non-parametic Kruskal–Wallis analyses of variance showed that the variables LL, MDS, MIBW, MLTR, MLW, MMBL, MMBW, MNSC, MOBL, MOBW, PL, SHFR, SH, SHFRA and SL were significant. Hence, these variables were used to distinguish the

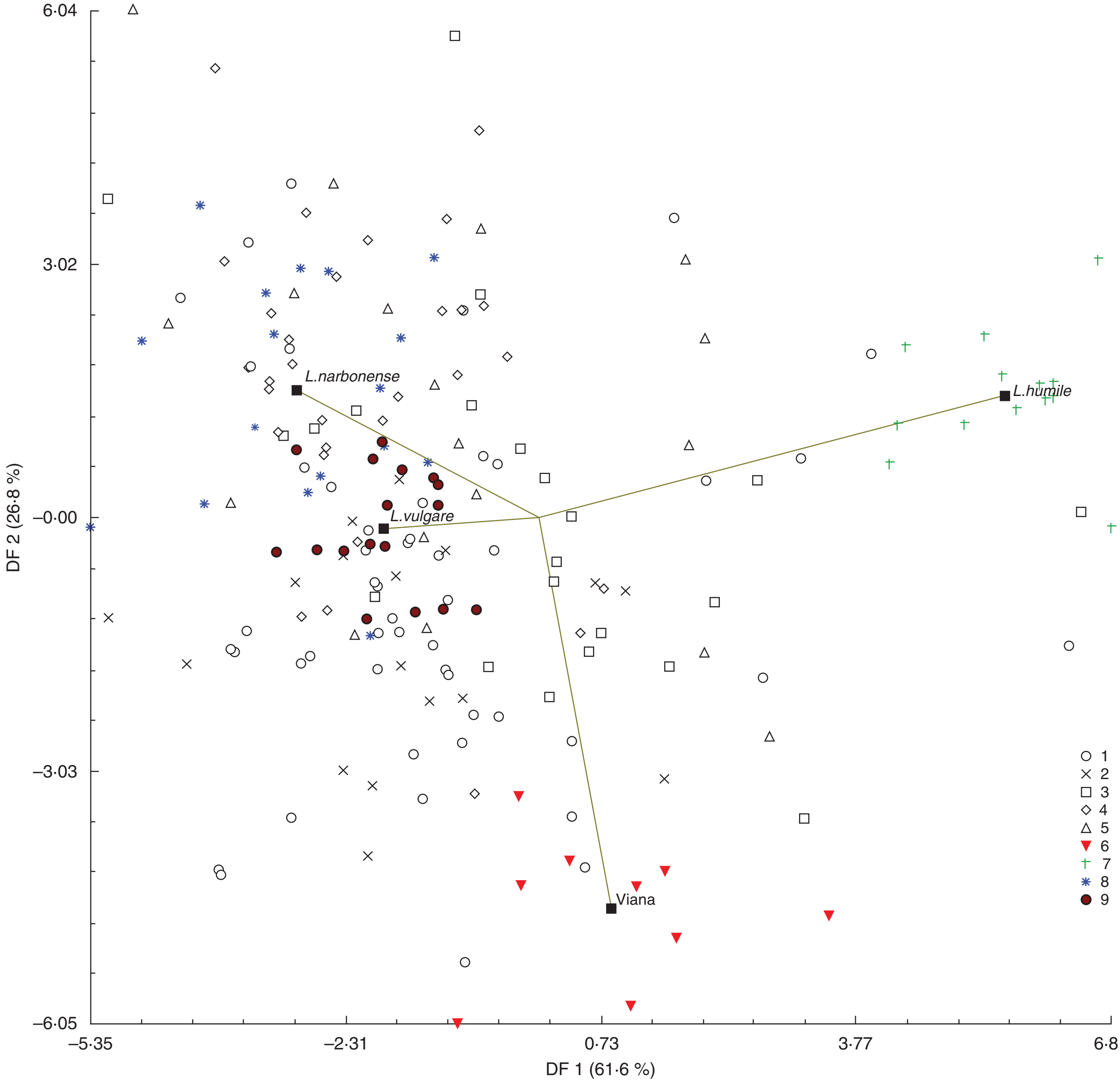

FIG. 3. Canonical discriminant function analyses of morphometric data with pre-defined *Limonium humile*, *L. narbonense* and *L. vulgare* species and specimens from the Viana population. Individuals from each species are represented by coloured symbols. Each species centroid is represented by filled squares. Percentages of total variance explained by the functions are given in parentheses. 1: Aveiro; 2: Mira; 3: Sado; 4: Tejo; 5: Formosa; 6: Praia da Veiga, Viana do Castelo; 7: *L. humile*; 8: *L. narbonense*; 9: *L. vulgare*.

reference species and populations studied. The reference species were discriminated by LL, MDS, MCL, MIBL, MLTR, MLW, MMBL, MMBW, MNSC, MOBL, MOBW, MSL, PL, SH, SHFR and SHFRA (Supplementary Data Table S1). In terms of morphometric variation, SH and SHFR were the most variable characters.

Principal component analysis based on morphometric traits revealed that the accumulated percentage of explained variance was 47·99 %, considering the first three principal components (after applying the 'broken stick model' criteria) (Fig. 2). The eigenvalues provided in Supplementary Data Table S2 showed that the amount of variance associated with the first dimension (PC1) was 21·27 % explained by a set of positively correlated variables LL, MLW, SL, SH, and SHFR; 16·74 % in the second dimension (PC2), establishing a gradient between SFRA (positive portion) and MOBL (negative portion) which was positively correlated with MIBL, MMBL, MDS, MSL and MIBW; and 9·88 % in the third dimension (PC3), reflecting a gradient between MSL (positive portion) and MNSC (negative portion) and positively correlated with MMBW and SFRA. Visual inspection of the PCA showed that all reference specimens are positioned in the limits of the arrangement obtained, whereas most specimens from sampled populations were localized in the centre of this arrangement. Specimens from the V population were always represented in the negative portion of the three represented components.

Canonical discriminant analysis of the reference species *L. vulgare*, *L. humile* and *L. narbonense* was perfect for discriminating them (first function 88 %, Wilks's lambda = 0·011; P = 0·000; second function 12 %, Wilks's lambda = 0·251; P = 0·000; Supplementary Data Fig. S1, Tables S3 and S4). The first dimension distinguished *L. humile* from the other reference species by the characters MIBL, MIBW, MNSC, MOBL and MSL. The second dimension separated *L. vulgare* from *L. narbonense* by the rest of the morphometric variables. Since in the PCA, V emerged as an isolated population (highlighted in the PCA; Fig. 2), we performed a second CDA to confirm if V individuals were discriminated into a new group. Thus, when using the three reference species and the V population as references, CDA accounted for 100 % of the variation (62·41 % in the first dimension, 26·04 % in the second dimension and 11·56 % in the third dimension) and correctly assigned individuals to species in 100 % of the cases ($n = 197$) (Fig. 3; Tables 4 and 5). The first dimension distinguished *L. humile* from the rest of the reference species and the V population by the characters MDS, MIBL, MLTR, MOBL, MNSC and MSL. The second dimension separated the reference species from specimens of the V population through variables LL, MIBW, MLW, SFRA, SH, SHFR and SL. In this last CDA, 30 individuals were classified in the V group (Tables 4 and 5). Remarkably, most populations were mixed, with *L. vulgare* being dominant in A (52 %) and F (88 %), and *L. narbonense* in M (50 %), S (63 %) and T (70 %). On the other hand, V appeared to be a pure population constituted by V specimens only.

Floral dimorphic vs. monomorphic populations

Heterostyly analyses revealed that most specimens were heterostylic (75·26 %; Fig. 4A, Table 6). Correspondence analysis of heterostyly–homostyly variables showed that heterostylous and homostylous individuals were significantly and heterogeneously distributed among populations ($\chi^2 = 25{\cdot}030$; $P = 0{\cdot}0053$). The first dimension of CA represented 88·93 % of explained variation, while the second dimension accounted for 11·07 % of the variation. A *pin* flower was the most frequent morphotype in the majority of populations ($\chi^2 = 17{\cdot}601$; $P = 0{\cdot}0034$). The V population differed significantly from the rest of the populations since most specimens showed homostylous flowers ($\chi^2 = 18{\cdot}450$; $P < 0{\cdot}001$).

TABLE 4. *Summary of the canonical discriminant analysis of* L. humile, L. narbonense, L. vulgare, *Viana specimens and individuals collected in natural populations*

Original group	Predicted group membership classification results				Total
	L. humile	*L. narbonense*	*L. vulgare*	Viana	
L. humile	13	0	0	0	13
L. narbonense	0	16	1	0	17
L. vulgare	0	0	17	0	17
Aveiro	5	11	27	9	52
Formosa	0	0	15	2	17
Mira	2	11	3	6	22
Sado	0	19	9	2	30
Tejo	3	14	1	2	20
Viana	0	0	0	9	9

TABLE 5. *Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions of morphological characters*

Morphometric variable	Function		
	1	2	3
LL	0·054	0·266*	−0·117
MCL	−0·089	−0·047	0·324*
MDS	−0·348*	0·197	−0·239
MIBL	−0·192*	−0·093	0·082
MIBW	−0·091	0·131*	0·012
MLTR	−0·157*	−0·017	−0·119
MLW	0·047	0·252*	−0·191
MMBL	−0·161	−0·157	−0·165*
MMBW	−0·034	0·088	−0·249*
MNFS	−0·039	−0·186*	−0·179
MNSC	0·428*	0·053	−0·121
MOBL	−0·150*	−0·060	−0·060
MOBW	−0·099	0·031	−0·133*
MSL	−0·541*	0·318	−0·211
PL	−0·040	−0·011	0·527*
SFRA	0·216	0·325*	−0·235
SH	−0·084	0·468*	−0·236
SHFR	−0·129	0·366*	−0·034
SL	−0·044	0·258*	0·017

Variables were ordered by absolute size of correlation within function (*). Details of acronyms are given in table 2.

Pollen–stigma analyses revealed that B was the most frequent combination (62·89 %; $\chi^2 = 16{\cdot}064$; $P = 0{\cdot}0067$). Only one individual exhibited flowers with combination C (Fig. 4B, Table 6). Correspondence analysis of pollen–stigma combination data showed that they were independently, non-significantly

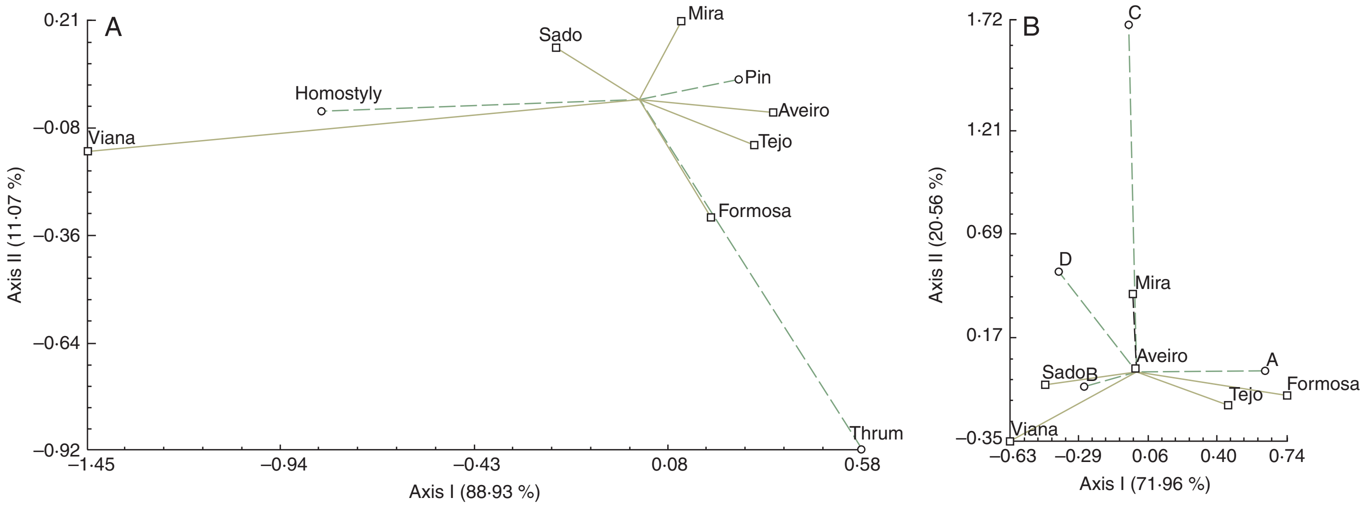

FIG. 4. The first two axes of correspondence analysis based on heterostyly and homostyly (A), and on pollen–stigma combinations (B). Percentages of total variance explained by the functions are given in parentheses.

TABLE 6. *Frequencies (%) of floral heteromorphies in* Limonium *specimens collected in natural populations*

Population	*n*	Heterostyly (%)		Homostyly (%)	Pollen–stigma combinations (%)			
		Pin	*Thrum*		A	B	C	D
Aveiro	20	17·53	1·03	2·06	6·19	12·37	0	2·06
Formosa	11	8·25	1·03	2·06	7·22	4·12	0	0
Mira	21	17·53	0	4·12	6·19	12·37	1·03	2·06
Sado	21	14·43	0	7·22	2·06	17·53	0	2·06
Tejo	16	13·4	1·03	2·06	8·25	8·25	0	0
Viana	8	1·03	0	7·22	0	8·25	0	0
Total	97	72·16	3·09	24·74	29·9	62·89	1·03	6·19

n, number of individuals analysed.

distributed among populations ($\chi^2 = 23{\cdot}314$; $P = 0{\cdot}0777$). The first dimension of CA represented 71·96 % of the variation, the second dimension 20·56 %, and the third dimension 7·48 %.

Estimations of seed set revealed that representative individuals from most populations showed that each plant produced >100 seeds per scape which had a low percentage of germination (26·5 %). This was not the case for specimens from the V population in which only two seeds were found, even after three visits in consecutive years (2011, 2012 and 2013) during the fructifying time (mid-July to the end of August). These two seeds, although they appeared to be mature, did not germinate.

Karyotype analysis revealed that most individuals from the A and T populations had $2n = 4x = 36$ chromosomes, but individuals with $2n = 4x = 35$ and 38 chromosomes were also found (Fig. 5). In most metaphase cells, four pairs of large sub-metacentric chromosomes, four pairs of medium sub-metacentric chromosomes, three pairs of medium metacentric chromosomes and 14 small metacentric or telocentric chromosomes were exhibited.

DISCUSSION

Taxonomy has a significant role in delineation and protection of biodiversity (Domínguez Lozano *et al.*, 2007). Studies based on herbarium data provide valuable information that is especially relevant for conservation purposes (Kricsfalusys and Trevisan, 2014) as they allow clarification of species distribution ranges and delimitate ambiguous species, which is the case for *Limonium* spp. TCG (Ennos *et al.*, 2005). In the study presented here, morphological and reproductive differentiation is found in closely related taxa *L. vulgare*, *L. humile* and *L. narbonense* from the Iberian coasts.

Our results confirm that there is too little variation in morphological descriptive variables to differentiate specimens from distinct populations. Instead, it is clear that morphometric data allow better discrimination between species than morphological variables. Based on a comprehensive revision of species in south-west Europe (Erben, 1978), morphometric data have been employed to examine several species with the same ploidy level, such as the tetraploid species of the *L. binervosum* complex from western Europe (Ingrouille and Stace, 1986), or species with distinct ploidy levels such as tetraploid *L. vulgare* and hexaploid *L. humile* in the British Isles (Dawson and Ingrouille, 1995), and diploid and tetraploid species from *L. ovalifolium* and *L. binervosum* complexes in Portugal (Róis *et al.*, 2013), or intraspecific variation in *L. dufourii* from eastern Spain (Rodríguez *et al.*, 2003).

Data from PCA using morphometric variables reveal that most specimens show similarities with the species used as a reference,

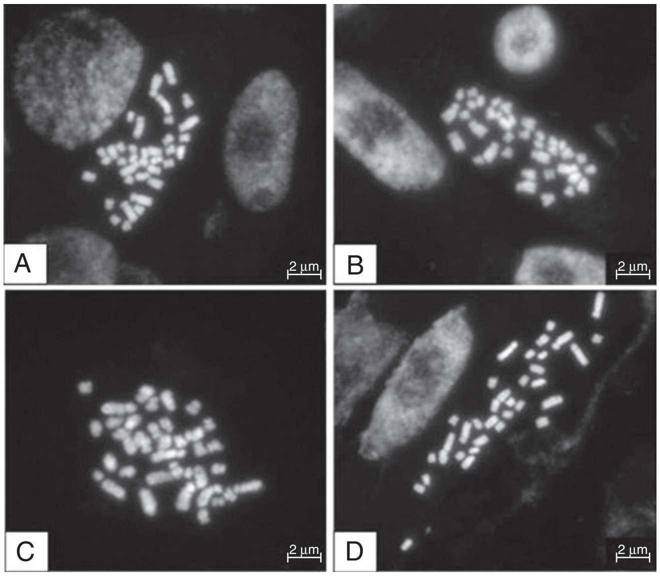

FIG. 5. Mitotic metaphase plates of DAPI-stained metaphase spreads from distinct individuals of Aveiro and Tejo populations. (A, B) Diploid individuals with $2n = 4x = 36$. (C, D) Aneuploid individuals with $2n = 4x = 38$ or $2n = 4x = 35$ chromosomes.

except for those from the V population. In the three represented components, these last (V) specimens were always separated from the first ones. Data from CDA using reference species revealed that *L. vulgare* specimens show higher morphological affinity with *L. narbonense* specimens than with those of *L. humile*. These first two species were discriminated from *L. humile* based primarily on the use of five morphometric variables, i.e. MIBL, MIBW, MNSC, MOBL, and MSL (first dimension). *Limonium vulgare* and *L. narbonense* separate from each other based on the remaining 14 morphometric variables (second dimension). In descriptions on Flora Iberica by Erben (1993), the variables MNSC and MLTR are not used to classify *L. humile*. Also, Erben (1993) did not use the variables MNFS and PL to discriminate *L. vulgare* from *L. narbonense*, which we found to be important in the present study.

Remarkably, in the CDA using the three reference species and the V population, a few specimens classified as *L. vulgare* in the first CDA (three reference species) are assigned to *L. narbonense*, supporting the view that these two species are morphologically very close. Previous studies have also suggested that these two species are morphologically (Erben, 1993) and phylogenetically (Palacios *et al*., 2000; Lledó *et al.*, 2005) close, although they do not share habitats in eastern Spain. Our results demonstrate, for the first time, that *L. narbonense* grows together with *L. vulgare* and *L. humile* in Portuguese coasts and that the first two species are prevalent in most populations. Although *L. narbonense* is considered an unresolved name in the Plant List (http://www.theplantlist.org/), this species has been a focus of taxonomic, cytological, breeding system and genetic studies (Erben, 1993; Crespo and Lledó, 1998; Lledó *et al.*, 2005; Georgakopoulou *et al.*, 2006; Palop-Esteban *et al.*, 2011). Confusion regarding its taxonomic identity is due to the numerous epithets which have been applied to this taxon (Erben, 1993; Crespo and Lledó, 1998). Although *L. narbonense* presents clear affinities with *L. vulgare* and *L. humile*, its taller size and distinct inflorescence structure allow an easy distinction in relation to these species. However, the V population differentiates from the three reference species by the morphometric variables LL, MIBW, MLW, SFRA, SH, SHFR and SL (Table 7).

TABLE 7. *Diagnostic morphometric characters among* Limonium maritimum *and its closest relatives* L. humile, L. narbonense *and* L. vulgare

	L. maritimum	*L. humile**	*L. narbonense**	*L. vulgare**
LL	2·7–6·4	6·2–26	7·0–27·5	3·5–17·0
MIBW	2·0–3·0	2·5–4·0	2·0–3·5	2–3·0
MLW	1·0–1·8	1·2–5·0	1·4–6·8	1·1–4·6
SFRA	0·1–0·4	0·2–0·5	0·4–1·0	0·3–0·7
SH	13·0–26·5	44·0–72·5	24·5–92·0	14·5–57·5
SHFR	5·4–9·0	19·0–40·0	6·0–40·3	6·0–36·0
SL	0·5–3·0	2·5–16·0	2·0–17·0	2·0–19·0

Acronyms and units are given in Table 2.
*According to Erben (1993).

It is interesting to verify that the differential characteristics of specimens from the V population are not restricted to their geographical distribution, because specimens of other populations, in particular, Aveiro and Mira, show these characteristics. In fact, except for Viana, most populations are mixed in the sense that individuals from distinct species are present in the same site. This indicates that there is no correlation between taximetry and geographic distribution, as the closest populations are not necessarily the most similar, nor are the populations furthest away the most distinct. In the morphometric studies by Dawson and Ingrouille (1995) for *L. humile* and *L. vulgare* in the British Isles, this situation was also observed.

Heterostyly, although not typical in *Limonium*, is present in *L. vulgare* (Baker, 1948, 1966), whereas pollen–stigma dimorphism associated with a sporophytic self-incompatibility system is common in *Limonium* spp. (Baker, 1966). Due to these flower heteromorphies which may facilitate cross-fertilization, *L. vulgare* and *L. narbonense* appear to be obligate outcrossers (Baker, 1948; Erben, 1979; Dawson and Ingrouille, 1995; Georgakopoulou *et al.*, 2006) whereas *L. humile* seems to be a facultative inbreeder (Dawson and Ingrouille, 1995). Here, analysis of flower heteromorphies in the collected specimens reveal that two flower morphs (*pin* and *thrum*) and pollen–stigma dimorphisms exist within most populations. Thus, self-incompatible individuals within each of these populations may cross-fertilize. Cytoembryological development studies show that meiotic (sexual) embryo sacs are formed in *L. vulgare* (D'Amato, 1940). Interestingly, at least in the British Isles, the first two species grow mixed together in some saltmarshes, and morphological variants can be found in some of these sites (Boorman, 1966, 1967; Dawson, 1990; Dawson and Ingrouille, 1995). In this study, we hypothesize that hybridization between *L. vulgare* and *L. narbonense* is possible due to their breeding systems. In fact, morphological hybrids *L. humile* × *L. vulgare* and *L. vulgare* × *L. narbonense* have been described (Erben, 1993). Also, in *L. vulgare* and *L. narbonense* mixed populations (e.g. A and T), balanced tetraploids ($2n = 4x = 36$) and unbalanced aneuploid tetraploids ($2n = 4x = 35$ and 38) are found. Therefore, we cannot

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Limonium maritimum Caperta, Cortinhas, Paes, Guara, Espírito Santo & Erben
Nº 1020/2013 Data 24-VIII-2013
Colect. Ana Caperta Det.
Local Mi; Viana do Castelo: Praia da Veiga, Areosa.
Alt. 0-1m UTM: 29TNG11
Obs. Em praias do Atlântico, crescente entre os afloramentos rochosos expostos a ondas na maré alta.

FIG. 6. Type specimen of *Limonium maritimum* Caperta, Cortinhas, Paes, Guara, Espírito-Santo and Erben.

exclude that at least some of these aneuploid individuals could reproduce through apomixis. In fact, in both triploid and tetraploid *Limonium* spp., apomitic embryo sacs have been observed (D'Amato, 1940, 1949). In contrast to specimens from other populations, V specimens only show homostylic flowers with a unique pollen–stigma morph (B). As this combination is self-incompatible, our results suggest that at least these individuals might reproduce through apomixis. Collectively, our morphometric and flower morph data help to delineate a new taxon in *Limonium* TCG, especially in *L. vulgare*-related species, widespread along the coast of Portugal, ***Limonium maritimum*** Caperta, Cortinhas, Paes, Guara, Espírito-Santo and Erben **sp. nova** (Table 7; Figs 6 and Fig. 7; Appendix 3).

In conclusion, for the morphologically closely related *Limonium* taxa studied, here our data suggested that the species are relatively new and evolving. Further work should be focused on: (1) anatomical and physiological traits that can be used as taxonomical characters; (2) molecular studies in order

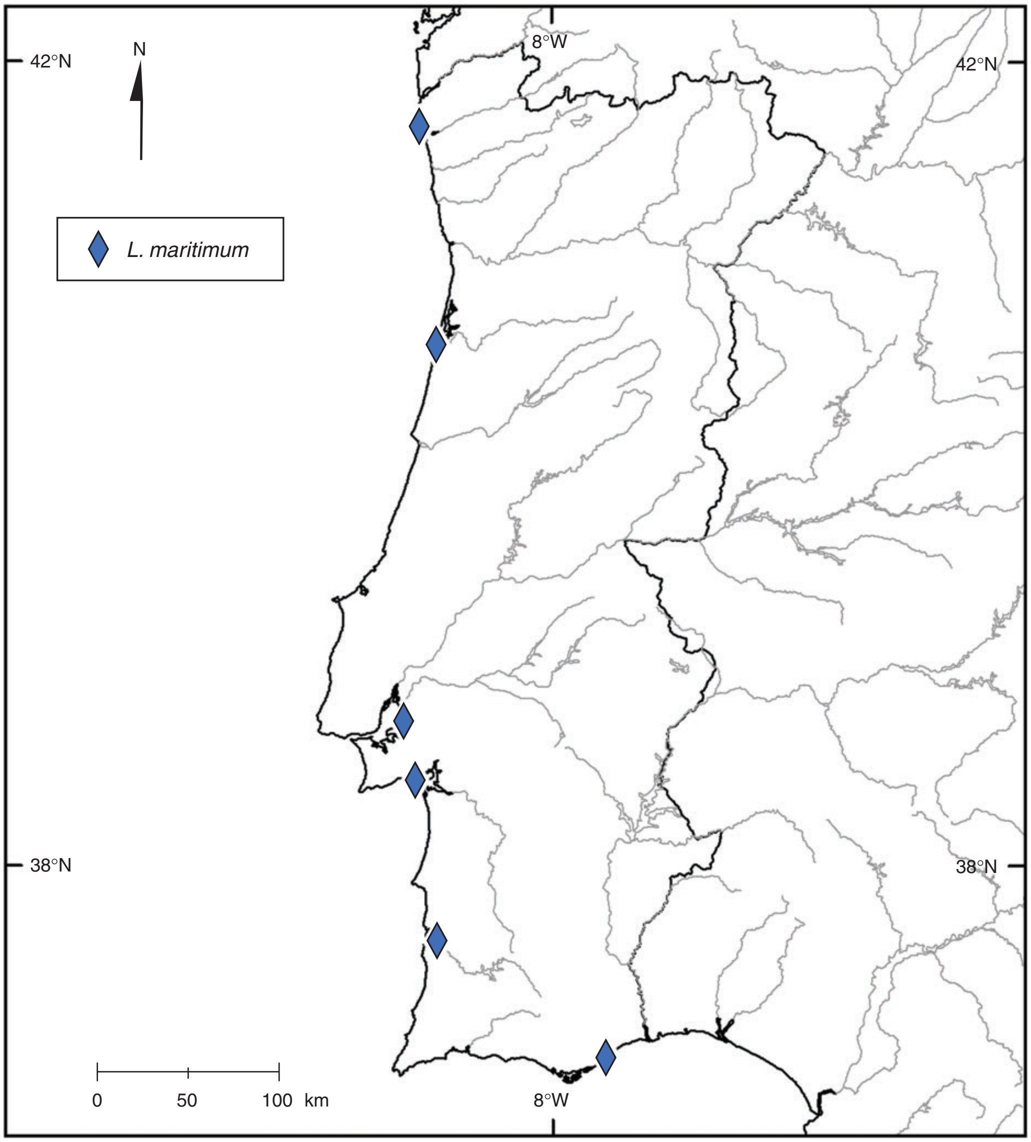

FIG. 7. Distribution map of *Limonium maritimum*. Rhomboids in blue represent populations in which *L. maritimum* individuals are observed.

to differentiate these species genetically; and (3) experimental controlled pollinations to provide evidence for potential hybridization between these taxa.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: mean values of morphometric characters in reference specimens *Limonium humile*, *L. narbonense* and *L. vulgare*, and collected specimens from natural populations. Table S2: Eingenvalues and percentages of variance associated with the first three principal components. Table S3: summary of the canonical discriminant analysis of *Limonium* spp. reference specimens, and collected specimens from natural populations. Table S4: pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions of morphological characters. Figure S1: canonical discriminant function analyses of morphometric data with pre-defined *Limonium humile*, *L. narbonense* and *L. vulgare* reference species.

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manuscript. D.E.S. revised the manuscript. All authors read and approved the manuscript. The authors declare that they have no conflicts of interest.

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APPENDIX

Appendix 1 *List of specimens used as reference species with voucher sample details*

Species	Herbarium number	Site location/Country	Collection date	Collectors
L. humile	ARAN 36106	Euskal Herria, Gipuzkoa, Zarautz/Spain	13–09–1986	P. Garín
	ARAN 9794	Euskal Herria, Gipuzkoa, Zarautz, Desembocadura de Iñurritza erreka/Spain	05–09–1982	Mª Salaverría
	ARAN 36107	Cantabria, San Vicente de la Barquera/Spain	21–08–1984	P. Catalán
	ARAN 52971	Euskal Herria, Pyrénées Atlantiques, Baiona, St. Bernard/Spain	24–09–1995	I. Aizpuru
	ARAN 36105	Cantabria, Argoños, Ancillo/Spain	29–09–1985	Aizpuru, Catalán and Aedo
	ARAN 55151	Euskal Herria, Gipuzkoa, Zumaia/Spain	07–08–1987	J. Elorza
	ARAN 47391	Euskal Herria, Gipuzkoa, Zumaia, Playa de Santiago/Spain	26–07–1989	J. L. Terés
	ARAN 47390			
	ARAN 9793	Euskal Herria, Gipuzkoa, Zarautz, Desembocadura de Iñurritza erreka/Spain	24–07–1981	Lizaur y Salaverría
	MA 617149	Cantabria: marisma de Rubín, junto a Abaño, San Vicente de la Barquera/Spain	23–07–1983	C. Aedo
	MA 681337	Miengo, Mogro/Spain	15–09–1995	NA*
	MA 681334	Cicero (Bárcena de Cicero)/Spain	29–08–1985	Herrá y Loricente
	MA 681335	Euskal Herria, Gipuzkoa, Zarautz/Spain	06–07–1973	NA
L. narbonense	MA 695629	Ayamonte/Spain	25–09–2011	E. Sánchez Gullón
	MA 394690	Castellón, Oropesa del Mar: Punta del Faro/Spain	08–08–1984	García-Villaraco
	MA 426143	Valencia: El Saler/Spain	28–10–1981	J. B. Peris
	VAL149647	Alicante: Pego (la marina alta)/Spain	08–12–1990	R. Pérez
	VAL149560	Alicante: Dénia (la marina alta)/Spain	27–07–1991	
	VAL149649	Alicante: Pego (la marina alta)/Spain	08–10–1987	
	VAL 20801	Valencia: Marjal de Oliva/Spain	14–09–1987	Pilar Soriano
	VAL 9382	Castellón: Cabanes (la plana alta), Torrelasal/Spain	07–08–1981	NA
	VAL19384	Castellón: Orpesa (Laplana Alta), Morro de Gós/Spain	09–09–1989	J. Tirado and C. Villaescusa
	VAL 19383	Castelló: Cabanes (La plana Alta), Platja Torrelasal/Spain	15–06–1989	
	VAL187540	Castelló: Xilxes (La Plana Baixa)/Spain	01–11–1993	A. Olivares
	VAL 21202	Castelló: Orpesa (La plana alta), Far d' Orpesa/Spain	27–07–1988	A. Aguilella, I. Baeza and J. Riera
	VAL 19385	Castelló: Castelló de la Plana (La Plana Alta), Aerodromo/Spain	09–09–1989	NA
L. narbonense	VAL 27396	Castelló: Torreblanca (La Plana Alta), Colt de Tomàs / Spain	25–08–1992	J. Tirado & C. Villaescusa,
	VAL 27372			
	VAL 28341	Castelló: Orpesa (La Plana Alta)/Spain	27–08–1992	
	VAL 19386	Castelló: Alcalá de Xivert (El Baix Maestrat) Cap i Corp / Spain	04–05–1991	Villaescusa & Tirado
	VAL 172185	Tarragona: Tamarit de Mar, La Mora / Spain	18–09–1948	C. Aedo
L. vulgare	MA 348673	La Coruña: Carnota, playa de Carnota / Spain	10–08–1982	S. Castroviejo
	MA 459699	La Coruña: Carballo, Playa de Baldayo / Spain	29–07–1987	E. Lago & C. Ferreiro, A. Paz
	MA 595187	La Coruña: Carnota, marismas de Carnota / Spain	30–07–1995	R.I.Louzán
	MA 470215	Vizcaya: Ria de Guernica, marismas de Axpe/Spain	08–08–1946	NA*
	MA 681330	Soano (Arnuero)/Spain	07–08–1981	NA
	MA 593015	Ria de Soano a Quejo/Spain	06–08–1990	C. Aseguinolaza and P. M. Echebarria
	MA 289828	Algarve: Ilha de Tavira/Portugal	20–04–1920	Malato Belizetal
	MA 371528	Beja, Vila Nova Mil Fontes, Rio Mira/Portugal	23–04–1984	M. Luceño
	MA 310871	Beira Litoral: Bunheiro: Bestida/Portugal	10–07–1975	M. Beliz and J. A. Guerra
	MA 372195	Aveiro, Ria de Aveiro, Murtosa/Portugal	20–08–1984	M. Luceño
	MA 372194			
	MA 289830	Beira Litoral: Bunheiro: Bestida/Portugal	10–07–1975	M. Beliz and J. A. Guerra
	VAL 154052	NA/UK	1827	Watson
	VAL 154054	Nord Beveland, Kamperland/The Netherlands	September 31	NA
	VAL 154053	Gironde, Arcachon/France	August 30	NA
	VAL 973674	Cantabria, Oriñon, Ría de Oriñón/Spain	14–09–1991	J. Aparício, T. Pérez and P. Urrutia

*NA, voucher without details.

APPENDIX 2. *List of taxa sampled in Portugal with voucher herbarium specimen details*

Herbarium	Herbarium number	Site location	Collection date	Collectors	Species[†]
Herbário da Estação Agronómica Nacional – LISE	LISE – 45358	Aveiro, Murtosa	23–08–1954	Bento V. Rainha	*L. maritimum*
	LISE – 6588	Aveiro, Brunheiro	11–09–1939	A. R. Pinto da Silva	*L. narbonense*
	LISE – 4730	Aveiro, Barra de Aveiro	27–07–1938	W. Rothmaler	*L. maritimum*
	LISE – 40159	Póvoa de Santa Iria	26–08–1942	C. Fontes and Manuel da Silva	*L. narbonense*
	LISE – 40625	Sacavém	16–09–1942	Manuel da Silva	*L. narbonense*
	LISE – 58240	Barreiro	23–07–1958	Bento V. Rainha	*L. narbonense*
	LISE-94940	Setúbal, rio Sado	15–7–1977	Manuel da Silva	*L. vulgare*
	LISE-5145	Setúbal, Península	17–9–1938	W. Rothmaler	*L. maritimum*
	LISE – 94568	Ilha da Armona	13–7–1929	M. H. Ramos Lopes	*L. vulgare*
	LISE – 92632	Faro, Ilha das Lebres	6–1916	F. Mendes	*L. maritimum*
Herbário João de Carvalho e Vasconcellos do Instituto Superior de Agronomia – LISI	LISI – 41897/1999	Sé, ría Formosa	08–07–1982	J. G. Pedro and F. Nascimento	*L. maritimum*
	LISI – 46618/1999	NA*	15–07–1988	José Carlos Costa	*L. vulgare*
	LISI – 348/2010	Quarteira, Praia do Ancão	17–10–2009	A. Caperta and A. R. Antunes	*L. maritimum*
	LISI – 46188/1999	Panasqueira	13–05–1988	José Carlos Costa	*L. narbonense*
	LISI – 47146/1999	Ilha da Armona	05–08–1988		*L. narbonense*
	LISI – 46148/1999	Fuzeta, Bias do Sul	11–05–1988		*L. narbonense*
	LISI – 47129/1999	Conceição, Cabanas de Tavira, Ribeira do Almargem	04–08–1988		*L. vulgare*
	LISI – 813/2010	Pedras D'el Rei	05–07–2010	A. Caperta, S. Róis and A. Paes	*L. maritimum*
	LISI – 259/2010		16–05–2010	A. Caperta and S. Róis	*L. maritimum*
	LISI – 552/2010		05–07–2010	A. Caperta, S. Róis and A. Paes	*L. vulgare*
	LISI – 553/2010				*L. vulgare*
	LISI – 554/2010				*L. vulgare*
	LISI-41781/1999	Reserva Natural de Castro Marim,Venta-Moinhos	22–06–1982	M. Lousã	*L. narbonense*
	LISI-36273/1999	Reserva Natural de Castro Marim	11–07–1978		*L. narbonense*
Herbário do Museu Nacional de História Natural – LISU	LISU – 150927	Ribatejo, Pancas,ríoSorraia	08–07–1982	M. Correia, H. Bacelar and J. Cardoso	*L. narbonense*
	LISU – 138887	Ribatejo, Pancas	30–09–1982	J. Alves and C. Duarte	*L. maritimum*
	LISU – 29143	Alcochete	09–1883	Pereira Coutinho	*L. narbonense*
	LISU – 29148	Barreiro	09–1883	A. R. da Cunha	*L. vulgare*
	LISU – 29146	Alcácer do Sal	15–09–1980	J. Daveau	*L. narbonense*
	LISU – 139914	Faro, Ilha das Lebres	06–1916	F. Mendes	*L. vulgare*
	LISU – 29150		06–1916	F. Mendes	*L. narbonense*
Herbário da Universidade de Coimbra – COI	COI – 20	Vagos	09–06–1961	J. Paiva	*L. maritimum*
	COI – 217	Ria de Aveiro, Barra de Aveiro	04–07–1967	J. Ormonde & R. Rodrigues	*L. vulgare*
	COI – 1030	Aveiro, Murtosa	1978	A.Marques	*L. vulgare*
	COI – 1340	Aveiro, Gafanha da Encarnação	18–06–1979	A.Marques	*L. vulgare*
	COI – 1491	Aveiro, Murtosa	03–12–1979	A.Marques	*L. narbonense*
	COI – 13866	Aveiro, Barra de Aveiro	27–07–1938	W. Rothmaler	*L. vulgare*
	COI – 13759	Aveiro	29–07–1976	Alexandrino Matos, Manuel & Alves	*L. vulgare*
	COI – 845	Sacavém	NA	NA	*L. maritimum*
	COI – 66	Setúbal, Pântanos da Cotovia	08–1900	A.Luisier	*L. vulgare*
	NA*		09–1906	A.Luisier	*L. narbonense*
Herbário da Universidade do Porto – PO	PO – 6448 G.S.	Estarreja	08–1984	A. Egas Moniz	*L. vulgare*
	PO – 6449 G.S.	Ria de Aveiro	08–1898	Gonçalo Sampaio	*L. maritimum*
	PO – 6450 G.S.	Ílhavo	30–07–1901		*L. vulgare*
	PO- 6451 G.S.	Ria de Aveiro: Gafanha	09–1898		*L. vulgare*
	PO – 52837	Ílhavo	21–7–1987	A. Serra, Armando and Loureiro	*L. maritimum*
	PO – 18798	Ovar: Marinha	30–11–1958	Martins d'Alte and G. Costa	*L. narbonense*
	PO – 18801	Ílhavo	27–06–1964	A. Rozeira and G. Costa	*L. narbonense*
	PO – 18802	Ria de Aveiro	28–07–1964	A. Rozeira	*L. maritimum*
	PO – 18803	Ílhavo: Gafanha da Nazaré	16–08–1965	A. Rozeira	*L. maritimum*
	PO – 18799	Faro, Salinas	17–06–1962	A. Rozeira, K. Koepp and G. Costa	*L. vulgare*

*NA, voucher without details.

[†] Classification results after canonical discriminant analysis.

Appendix 3 Description of ***Limonium maritimum*** Caperta, Cortinhas, Paes, Guara, Espírito-Santo and Erben **sp. nov.**

Type: Portugal, Mi; Viana do Castelo: Praia da Veiga, Areosa. Alt. 0–1 m, UTM: 29 T NG11, 24–08–2013, *Ana Caperta* (holotype: LISI-1020/2013, isotype: Herb. Erben)

Planta perennis, glabra, viridis, 13–26·5 cm alta, foliis 2·7–6·4 × 1–1·8 cm, oblanceolatis, apice obtusis ad acutis, aristatis, pinnatis, caulibus paucis, ramis sterilibus absentibus vel paucissimis, spicis 0·7–4·0 cm longis, spiculis (1-) 2–3-floris, remotis ad 4–5 in 1 centimetro dispositis, bractea inferiore 2·0–3·5 × 1·0–2·0 mm, tiangulari-ovata, bractea media 2·5–5·5 × 1·0–2·0 mm, anguste triangulari-ovata, bractea superiore 3·0–6·0 × 2·0–3·0 mm, anguste elliptica, mucronata, calyce obconico, 5·0–6·5 mm longo, tubo unilateraliter sparsim longe piloso. Corolla violacea.

Perennial plant, glabrous, forming a sub-shrub 13–26·5 cm tall, with few erect stems and a robust tap root. Caudices 8·0–15 cm long, branched, living leaves in rosettes at apices. Leaves fleshy, grey-green, glaucous, 2·7–6·4 × 1·0–1·8 cm, oblanceolate, apex obtuse at acute, mucronate, with one central nerve and some pinnately branching lateral nerves, gradually tapering into the petiole; the majority withered at anthesis. Stems 5·4–9·0 cm long, rugose, nearly straight to slightly flexuous, with branching normally in the middle of each of the stems. Inflorescence corymbose or obtrullate in outline. Sterile branches absent or very few. Fertile branches 7·6–17·5 cm long, straight, directed obliquely upwards, forming branching angels of 5–24 °, longer branches in the upper half divided. Spikes 0·7–4·0 cm long, straight, erect to directed obliquely upwards. Spikelets composed of (1-) 2–3 flowers, remotely arranged with 4–5 per cm, adpressed in the axis of spices. Outer bract 2·0–3·5 × 1·0–2·0 mm, triangular-ovate, acute, mucronate; bract margin membranous; central part fleshy, acuminate. Middle bract membranous, 2·5–5·5 × 1·0–2·0 mm, narrowly triangular-ovate. Inner bract 3·0–6·0 × 2·0–3·0 mm, narrowly elliptic, normally mucronate; bract margin broadly membranous; central part fleshy, green, oblong, acuminate. Calyx obconical, 5·0–6·5 mm long; calyx tube on one half sparsely, long hairy, with five ribs ending at the base of the lobes. Corolla violet.