

## *Limonium silvestrei* (Plumbaginaceae), a new agamospecies from southern Spain

Abelardo Aparicio

*Departamento de Biología Vegetal y Ecología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain (e-mail: abelardo@us.es)*

*Received 2 Dec. 2004, revised version received 7 Mar. 2005, accepted 29 Mar. 2005*

Aparicio, A. 2005: *Limonium silvestrei* (Plumbaginaceae), a new agamospecies from southern Spain. — *Ann. Bot. Fennici* 42: 371–377.

*Limonium silvestrei* Aparicio (Plumbaginaceae) is described and illustrated from the only known population located within an agricultural landscape in southern Spain. Based in the single A pollen/Cob stigma combination, male sterility, jumbled 3x karyotype and high seed set and germination, *L. silvestrei* should be regarded as a new agamospecies in *Limonium*. The number of long metacentric chromosomes observed in the karyotype of this species is in conflict with Erben's theory about the evolution in the genus. The discovery and characterisation of this species stresses the relevance of forest fragments of natural vegetation in agricultural landscapes as biodiversity reservoirs.

Key words: agamospermy, forest fragmentation, hybridization, polyploidy

### Introduction

The genus *Limonium* has a world-wide distribution on coasts and inland, occupying salt or gypseous steppes and other arid environments. It comprises 400 species, out of which 107 species (87 endemic) and 137 hybrids are located in the Iberian Peninsula (Erben 1993). Moreover, it is considered that the western Mediterranean region represents a primary centre of evolution showing the highest taxonomic diversification and complexity in *Limonium* (Erben 1993).

A series of polyploids is recognized in Old World *Limonium*, ranging from  $2x = 12$  to  $6x = 54$  including diploids ( $2n = 12, 14, 16, 18$ ), triploids ( $2n = 3x = 25, 26, 27$ ), tetraploids ( $2n = 4x = 32, 34, 35, 36$ ), pentaploids ( $2n = 5x = 42, 43$ ) and hexaploids ( $2n = 6x = 54$ ). These

are geographically distributed in the western Mediterranean ( $3x$ ), eastern Mediterranean ( $5x, 6x$ ) and on the Atlantic coasts ( $4x$ ) (Erben 1979, Artelari & Kamari 1986, Cowan *et al.* 1998). A huge taxonomic complexity is also inherent in the genus, which Erben (1979) explained by means of an evolutionary model based upon chromosome rearrangements and hybridization. In summary, through several combinations of reduced and unreduced gametes from sexual species, odd sterile polyploids would be released and further maintained by agamospermy. Most authors have accepted this model involving hybridization and agamospermy as the evolutionary pathway in *Limonium* (e.g. Artelari & Kamari 1986, Cowan *et al.* 1998, Artelari & Georgiou 1999, 2002), although local evolution of seed clones through mutation and

chromosome rearrangements seems also central to the evolution of *Limonium* (Ingroulle & Stace 1985).

Specific agamosperous mechanisms such as diplospory or parthenogenesis have seldom been tested in *Limonium* (see Cowan *et al.* 1998); however, several authors have suspected agamospermy of providing the existence of (i) a unique self-incompatibility pollen/stigma combination within populations, (ii) generalized male sterility, (iii) odd polyploid chromosome number with jumbled karyotypes, and (iv) high seed productivity (Ingroulle & Stace 1985, Cowan *et al.* 1998). The agamosperous species so characterized account for a large number of species in the genus, and typically exhibit locally restricted distributions (Erben 1979, Ingroulle & Stace 1985).

As a result of a detailed inventory of fragments of natural vegetation in western Andalusia (southern Spain) (Aparicio *et al.* 2002), a *Limonium* population was collected and analysed in detail studying the morphology, breeding system, karyology and viability. In this article, a new species is described, and the relevance of forest fragments of natural vegetation as biodiversity reservoirs is also emphasised.

## Material and methods

Several herbarium specimens were collected from the population in June 2004. This population was visited in subsequent weeks for the description of the community, for estimations of population extension and size, and for collecting mature seeds and soil samples for further analysis.

### Breeding system

The existence of dimorphism in pollen and stigmas within population was investigated in flowers from ten herbarium specimens (20 flowers in total). The pollen grains and stigmas were removed and mounted in glycerol jelly stained with basic fuchsin, and observed under a light microscope at 400× magnification.

### Seed production and seed germination

To evaluate seed set in natural conditions, mature inflorescences of 14 plants were collected in the field, and 90 spikelets containing 192 flowers investigated for seed production. Likewise, to test germination capability, 175 seeds were placed in five Petri dishes (35 each) on a three-layer water moistened filter paper. Germinated seeds were daily counted and removed for further analysis.

### Karyology

Two–three-day-old seedlings from the previous experiment were treated with 0.002 M 8-hydroxyquinoline for about 3.5 h (Tjio & Levan 1950) at 4 °C, fixed in a mixture of 1:1:5 absolute ethanol, chloroform, acetic acid overnight, and stored at 4 °C in 70% ethanol. Root-tips were stained in cold alcoholic hydrochloric acid-carmin (Snow 1963) for 72 h at room temperature and then squashed in 45% acetic acid. Unambiguous metaphase plates were evaluated in as many cells and root-tips as possible. Images of the plates were taken and chromosomes described following the terminology of Levan *et al.* (1964) and Küppfer (1974). The categorisation of chromosomes follows the criteria in Stebbins (1938).

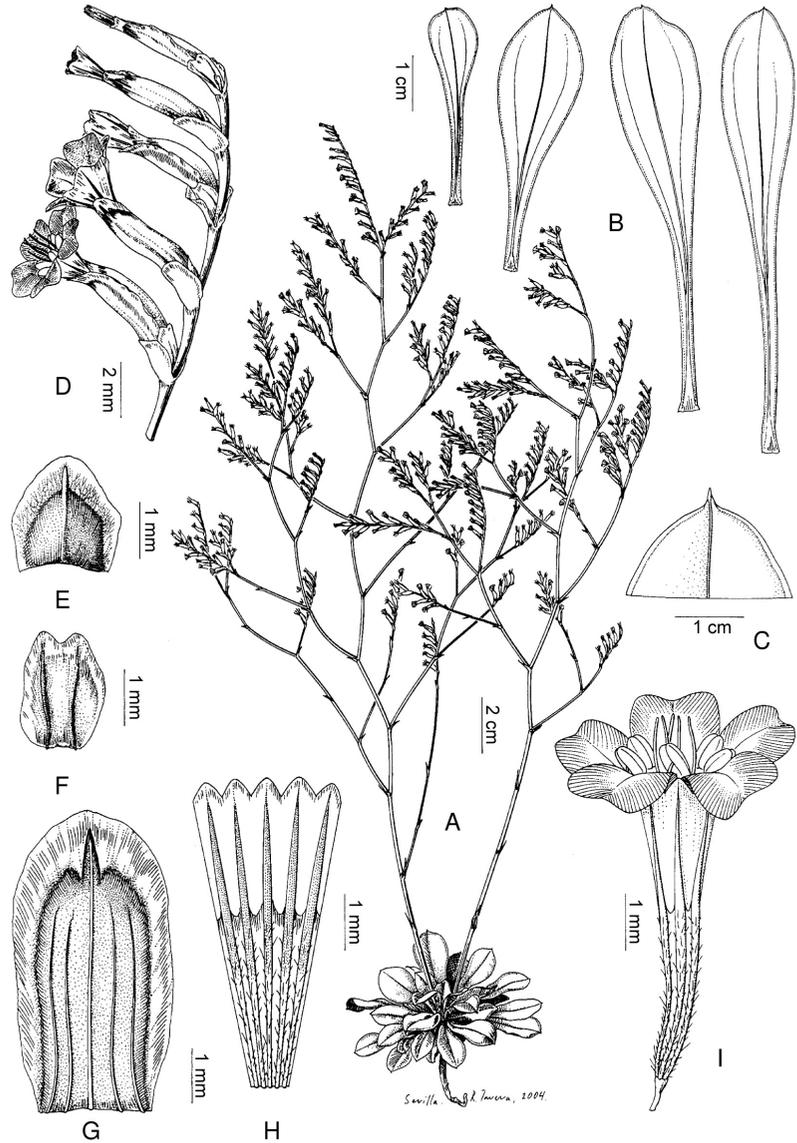
### Soil analysis

Three soil samples collected at a 0–5 cm depth from the population were pooled, and the pH in a deionised water saturated mash was determined employing a pH meter.

## Results

*Limonium silvestrei* Aparicio, *sp. nova* (Fig. 1)

*Species habitu L. virgatum* (Willd.) Fourr., *sed differt foliis 25–60(95) × 10–18(23) mm, spatulatis vel oblanceolatis, trinervis, apice acutiusculis vel rotundatis, mucronatis; inflorescentia 20–50(55) cm alta, ramis sterilibus deficientibus vel in parte inferior 1–2; bracteis exterioribus*



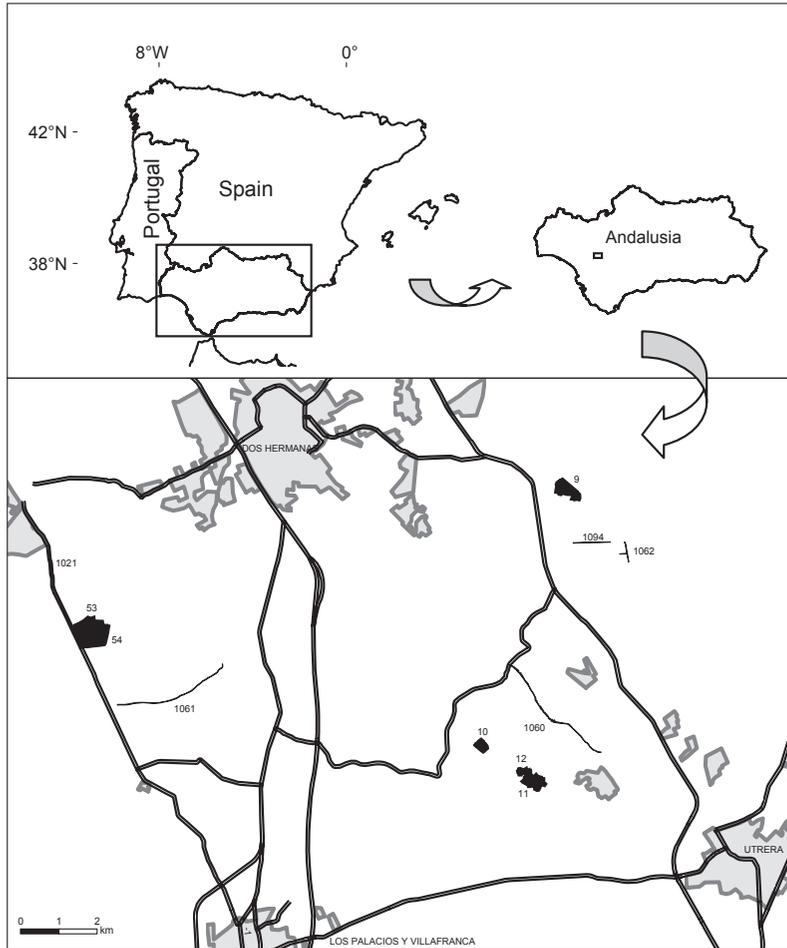
**Fig. 1.** *Limonium silvestrei* (from holotype). — **A:** Plant. — **B:** Leaves. — **C:** Leaf apex. — **D:** Detail of a branch of the inflorescence showing spikelets. — **E:** Outer bract. — **F:** Middle bract. — **G:** Inner bract. — **H:** Expanded calyx. — **I:** Flower.

(2)2.2–2.8(3) × 1.6–2.2 mm; flores 3–3.2 mm diametro.

TYPE: Spain. Utrera, sandy soils on Triassic marls and limestones in ‘Vereda de Dos Hermanas’, 13.V.2004 A. Aparicio (holotype MA; isotypes SEV, MAB).

Perennial with 1–4(6) glabrous, robust stems. Caudicles 0.5–3 cm densely spirally leafy. Basal leaves 2.5–6(9.5) × 1–1.8(2.3) cm, withered at anthesis, spatulate to oblanceolate, rounded to obtuse with a short mucro (0.1–0.4 mm), 3-veined; petiole 6–10 × 2–3(5) mm. Inflorescence

20–50(70) cm long, erect, paniculate, loosely branched from lower third; sterile branches absent or 1–2 in lower part. Branches of first order up to 16 cm long ± dichotomously divided, straight to arcuate, obliquely inserted (40°–60°), loosely branched. Spikes (11)14–65(80) mm, erect or arcuate. Spikelets 5.5–6.5(7) mm long, slightly curved, 2–3 per cm, including (1)2–4 flowers. Outer bract (2)2.2–2.8(3) × 1.6–2.2 mm, triangular-ovate, subacute, margin broadly membranous and central part slightly fleshy, apex nearly reaching margin. Middle bract 1.5–2 × 1.1–1.2 mm,



**Fig. 2.** Detail of the area where *Limonium silvestrei* was collected. The only known population spreads within the hedge number 1060. The numeration of patches of natural vegetation was the same as that from the original report by Aparicio *et al.* (2002).

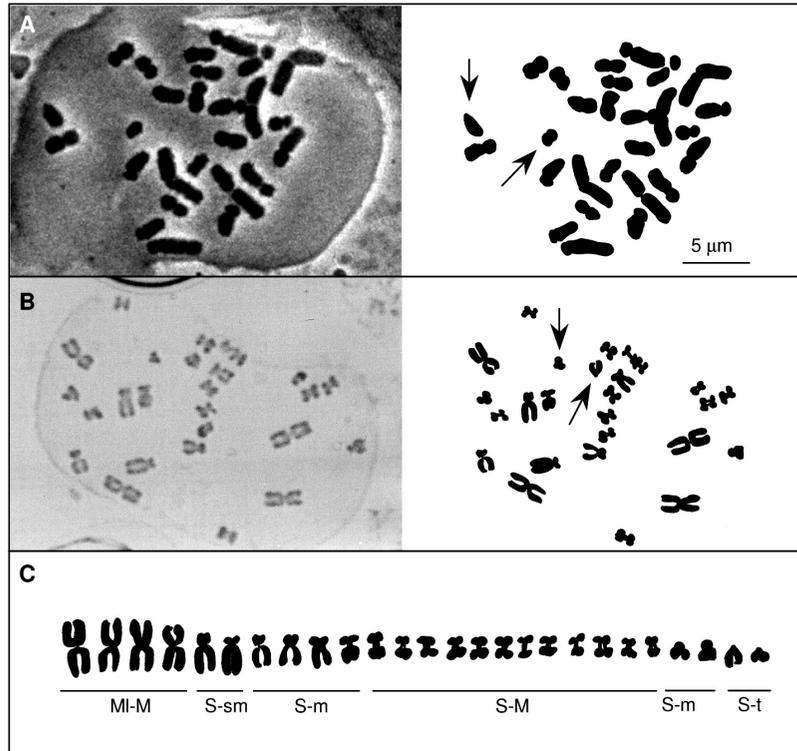
widely oblong-ovate, membranous. Inner bract  $4.5\text{--}6 \times 2.7\text{--}3.2$  mm, oblong-elliptic to oblong-ovate, obtuse to subobtuse, widely membranous in margin; central part  $3.5\text{--}5.5 \times 1.7\text{--}2.2$  mm, fleshy, oblong, with apex  $0.7\text{--}0.9 \times 0.2\text{--}0.3$  mm nearly reaching margin. Flowers  $3\text{--}3.2$  mm in diameter. Calyx  $5\text{--}6$  mm, overtopping inner bract by  $0.5\text{--}2.5$  mm; tube sparsely hairy on surface, hairs  $0.1\text{--}0.3$  mm; teeth  $0.3\text{--}0.5 \times 0.5\text{--}0.7$  mm, triangular-ovate; midribs ending at base of teeth. Petals  $7\text{--}8.5 \times 1.4\text{--}2.2$  mm, cuneate, pale violet. Stamens  $4\text{--}5.5$  mm, unequal; anthers  $0.4\text{--}0.7 \times 0.2$  mm. Stigma  $5\text{--}5.2$  mm, Cob-type, exerted, white. Fruit monospermous,  $3\text{--}3.3 \times 0.6\text{--}0.7$  mm, subcylindric, brown, with 5 slender ribs. Seed  $2.8\text{--}3 \times 0.6$  mm, smooth, red-brown, shiny.

**ETYMOLOGY.** The new species is named in honour of

Prof. Santiago Silvestre-Domingo, a colleague and an eminent botanist and plant taxonomist in Spain.

### Ecology, distribution and population size

*Limonium silvestrei* was collected in an area occupied by extensive cultivations of olives, oranges, sunflower, cotton, beetroot and orchards; however, small and scattered fragments of helm-oak, cork-oak, and *Pinus pinea* stands can still be found (Fig. 2). The hedge, number 1060 (numeration is the same to that from the original report by Aparicio *et al.* 2002), is about 3 km long and located at both sides of a frequently used track. Main woody species comprise *Pistacia lentiscus*, *Pinus pinea* accompanied by *Pinus halepensis*, *Quercus coccifera*, *Chamaerops humilis*, *Smilax aspera*, *Rhamnus lycioides* subsp. *oleoides*,



**Fig. 3.** Karyology of *Limonium silvestrei*. — **A** and **B**: Metaphase plates of two different plants showing the same jumbled karyotype. — **C**: Karyogram composed of 4 medium-large (MI) metacentric (M) chromosomes, 12 small (S) metacentric (M), 6 small metacentric (m), 2 small submetacentric (sm), and 2 small telocentric (t) (arrowheads).

*Retama sphaerocarpa*, *Teucrium fruticans*, *Osyris alba* and *Cistus albidus*, and *L. silvestrei* appeared restricted to the pasture, about 350 m along the hedge cohabiting with weed species such as *Cynurus echinatus*, *Elymus elongatus* subsp. *elongatus*, *Piptatherum multiflorum*, *Lagurus ovatus*, *Cynodon dactylon*, *Aster squamatus*, *Heliotropium europaeum*, *Plantago coronopus*, etc. The *L. silvestrei* community develops on sandy soils derived from alkali-rich Triassic marls with a high pH value (9.5). The population size of *L. silvestrei* was estimated to be about 2000 individual plants, most of which were able to bloom. In the field, the recruitment rate was apparently high.

### Breeding system

All ten studied plants in the population apparently had the self-incompatible A pollen/Cob stigma combination. However, it was remarkable that while stigmas were consistently Cob-type, pollen was found to be extremely variable in size, shape, number of colpi, stainability and degree of exine development.

### Seed production and germination

The mean number of flowers within a spikelet was  $2.14 \pm 0.75$  (range 1–4); likewise, the mean number of fruits per spikelet was  $1.35 \pm 0.69$  (range 0–3). The probability of finding a fruit within a spikelet was 0.90 and the overall probability of finding a fruit within a flower was 0.64. Seed germination took place between 1 and 6 days, and showed an overall germination percentage of 92%.

### Karyology

Clear metaphase plates were observed in ten root-tips, consistently showing the somatic chromosome number  $2n = 26$  (Fig. 3) with the following karyotype: 4 medium-large (MI) metacentric (M) chromosomes, 12 small (S) metacentric (M), 6 small metacentric (m), 2 small submetacentric (sm), and 2 small telocentric (t). The cytological technique and quality of plates did not provide enough information to unambiguously set chromosomes in homologous pairs,

but the jumbledness of the karyotype was at least demonstrated by the lack of homology between both t chromosomes.

## Discussion

The data presented here indicate that *L. silvestrei* is a highly fertile species in terms of seed set and seed germination. Furthermore, most flowers in every plant were able to set seed which then showed nearly full germination capability in the laboratory. Accordingly, many young plants could be found in the field, and success in seedling establishment also seemed to be high.

However, the rest of the biological data gathered in this study points to the existence of an asexual agamospermous breeding system in *L. silvestrei*. Firstly, most sexual *Limonium* species exhibit the dimorphism of pollen and stigmas associated with a self-incompatibility breeding system (Baker 1953); all the plants in the studied *L. silvestrei* population were apparently A/Cob, hence making the existence of sexuality for seed set unlikely. Secondly, male fertility must be reduced, as indicated by the irregular pollen grains yielded by microsporogenesis. The jumbled karyotype and the lack of full chromosome homology are central to the origin of male (and female) sterility in this agamospecies.

Once *L. silvestrei* was characterized as an agamospecies, the most relevant result in this study lies in the karyotype. The evolutionary model in the genus proposed by Erben (1979) states that polyploids would arise after hybridization through several combinations of reduced and unreduced gametes from sexual diploid species with  $n = 8$  and/or  $n = 9$  chromosomes. As a first step, descending aneuploidy would originate the  $2n = 16$  karyotype from the  $2n = 18$  karyotype (both including sexual species). Erben (1979) hypothesized that the 'long metacentric' chromosome observed in all  $2n = 16$  plants should be considered as a 'marker' chromosome for this complement. In this way, he stated that hybrid polyploids should bear as many 'marker' chromosomes as the  $x = 8$  complement would involve. Therefore, following Erben's model, tri-

ploids  $2n = 25$  ( $2 \times 8 + 1 \times 9$ ) should bear two long 'marker' metacentric chromosomes,  $2n = 26$  ( $1 \times 8 + 2 \times 9$ ) one, and  $2n = 27$  ( $9 \times 3$ ) none. This mode of evolution also accounts for higher polyploids and has been supported by many karyological studies (Erben 1979, Ingroulle & Stace 1985, Artelari & Kamari 1986, Artelari & Georgiou 1999, 2002, Sáez *et al.* 1998a, Roselló *et al.* 1998). However, Sáez *et al.* (1998b) found in *L. vigoi*, a  $2n = 36$  ( $4 \times 9$ ) tetraploid, an unexpected long metacentric chromosome. Likewise, in *L. silvestrei* four long metacentric chromosomes have been found, whereas just one would have been expected following the Erben's (1979) theory. In view of these results, the origin of *L. silvestrei* through direct hybridization between a reduced gamete  $n = 8$  and an unreduced gamete  $2n = 18$  can be ruled out. This fact supports the alternative evolutionary mechanism proposed by Ingroulle (1984) and Ingroulle and Stace (1985), as opposed to mere hybridization, such as somatic mutations, chromosome rearrangements and auto-segregation at a local scale, giving rise to morphologically and geographically identifiable clones of asexual agamospecies in *Limonium*.

Finally, most of the *Limonium* agamospecies are typically local endemics with restricted distribution and are usually regarded as 'microspecies' (Cowan *et al.* 1998). Furthermore, in Spain most of them are actually facing serious risk of extinction as evidenced by several red lists (Blanca *et al.* 2000, Bañares *et al.* 2003), where *Limonium* is the genus with the highest number of catalogued species. Out of the 107 Iberian species of *Limonium*, 23 (four sexual + 19 agamospecies) are included in the CR or EN categories (IUCN 2001) by Bañares *et al.* (2003). *Limonium silvestrei* should be immediately considered in the CR category, taking into account its population size and distribution, and the fact that its natural environment is continuously managed and its habitat actively degraded. The new description of this species, and previous data on endangered flora inhabiting forest fragments in southern Spain (Aparicio *et al.* 2002, Garrido *et al.* 2002), jointly highlight the relevance of forest patches of natural vegetation within agricultural landscapes as biodiversity reservoirs.

## Acknowledgements

The Andalusian Regional Government (Junta de Andalucía, Consejería de Medio Ambiente) funded the inventory of the 'island-forests' in western Andalusia. Final preparation of the manuscript was supported by grants from Junta de Andalucía (Plan Andaluz de Investigación), the Spanish Ministerio de Ciencia y Tecnología (project CGL2004-00022) and the Fundación BBVA (Banco Bilbao Vizcaya Argentaria). I am grateful to Prof. Dr. S. Silvestre-Domingo for providing the Latin diagnosis and the detailed description of this species.

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