

REVIEW

Secretory structures in plants: lessons from the Plumbaginaceae on their origin, evolution and roles in stress tolerance

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Abstract

The Plumbaginaceae (non-core Caryophyllales) is a family well known for species adapted to a wide range of arid and saline habitats. Of its salt-tolerant species, at least 45 are in the genus *Limonium*; two in each of *Aegialitis*, *Limoniastrum* and *Myriolimon*, and one each in *Psylliostachys*, *Armeria*, *Ceratostigma*, *Goniolimon* and *Plumbago*. All the halophytic members of the family have salt glands, which are also common in the closely related Tamaricaceae and Frankeniaceae. The halophytic species of the three families can secrete a range of ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , HCO_3^- , SO_4^{2-}) and other elements (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn). Salt glands are, however, absent in salt-tolerant members of the sister family Polygonaceae. We describe the structure of the salt glands in the three families and consider whether glands might have arisen as a means to avoid the toxicity of Na^+ and/or Cl^- or to regulate Ca^{2+} concentrations within the leaves. We conclude that the establishment of lineages with salt glands took place after the split between the Polygonaceae and its sister group the Plumbaginaceae.

KEYWORDS

anatomy, halophytes, phylogenetic analysis, physiology, salt glands

1 | INTRODUCTION

Exudation is a common phenomenon in plants and specialised tissues have evolved to secrete a variety of substances from nectar to mucilages to salts (Fahn, 1988; Lüttge, 1971). Glands that secrete salt are found across the families of flowering plants with a diversity of structures having evolved to fulfil this role (Dassanayake & Larkin, 2017; Thomson, Faraday, & Oross, 1988). Fahn (1988)

concluded that secretory tissues first arose as idioblasts scattered within tissues and these then developed to form ducts and cavities, finally evolving into secretory structures, the glandular trichomes. Here we examine the distribution of salt-secreting structures in the Plumbaginaceae and discuss our observation that all salt-tolerant species within the family utilize glands to secrete salt, whereas salt glands are absent in salt-tolerant members of the related Polygonaceae.

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The Caryophyllales is one of the major lineages of flowering plants with about 39 families and approximately 12,500 species (Bremer et al., 2009; Hernandez-Ledesma et al., 2015) and their phylogenetic relationships have been studied over a long time by morphology (Rodman, 1994), targeted gene sequencing (Brockington, Walker, Glover, Soltis, & Soltis, 2011; Rettig, Wilson, & Manhart, 1992; Schäferhoff et al., 2009), plastome sequencing (Arakaki et al., 2011) and transcriptome sequencing (Leebens-Mack et al., 2019; Walker et al., 2018; Yang et al., 2015). Based on such studies, the Angiosperm Phylogeny Group's classification, APG III (Bremer et al., 2009) and APG IV (Byng et al., 2016), support the expansion of the traditional Caryophyllales (i.e., corresponding essentially with the original Centrospermae) to include the noncore-Caryophyllales - the carnivorous families Droseraceae, Drosophyllaceae, Nepenthaceae, Ancistrocladaceae, Dioncophyllaceae, and allies Tamaricaceae, Frankeniaceae, Polygonaceae, and Plumbaginaceae (Brockington et al., 2009; Hernandez-Ledesma et al., 2015).

The Plumbaginaceae Juss. is a cosmopolitan family of perennial herbs, shrubs or small trees, rarely climbers (Kubitzki, 1993) that is well supported as monophyletic (e.g., Cuenoud et al., 2002; Hilu et al., 2003). The family is included in the Polygonids clade of the Caryophyllales (Figure 1 and Table S1), which comprises carnivorous (Ancistrocladaceae, Drosophyllaceae, Droseraceae, Nepenthaceae) and other non-carnivorous sister taxa (Frankeniaceae, Tamaricaceae, Plumbaginaceae and Polygonaceae). The Plumbaginaceae is comprised

of two subfamilies, the Plumbaginoideae and the Limonoideae. Genera of the Limonoideae are thought to have initially diversified in the Mediterranean and Irano-Turian regions, although a few genera also occur in the Southern Hemisphere (Lledó, Crespo, Fay, & Chase, 2005; Malekmohammadi, Akhiani, & Borsch, 2017; Moharrek et al., 2019; Table S2): *Aegialitis* is the only genus of the Limonoideae with a tropical distribution (two mangrove species in Asia and Oceania). Within the Plumbaginoideae, its members predominantly occur in arid and saline environments and often in coastal habitats (Hernandez-Ledesma et al., 2015; Kubitzki, 1993; Malekmohammadi et al., 2017; Moharrek et al., 2019). Of the nearly 940 species in the family as a whole, 5% display salt tolerance (Table S2), ranking the family fourth in a list based on the proportion of species within a family that are halophytes (Santos, Al-Azzawi, Aronson, & Flowers, 2016).

Halophytes are plants that survive in the presence of significant concentrations of soluble salt in the medium in which they grow. Quite what amounts to a 'significant concentration of salt' has been a matter of debate over the years (see, e.g., Breckle, 2002; Flowers & Colmer, 2008; Huchzermeyer & Flowers, 2013) with Aronson (1989) selecting a salt concentration of around 80 mM as the dividing line that separates halophytes from more salt-sensitive species, commonly called glycophytes. Flowers and Colmer (2008) used a higher salt concentration, of 200 mM, in order to discriminate higher levels of tolerance. However, even the ability to tolerate 80 mM NaCl is rare with fewer than 1,500 species having evolved this ability (<https://www.sussex.ac>).

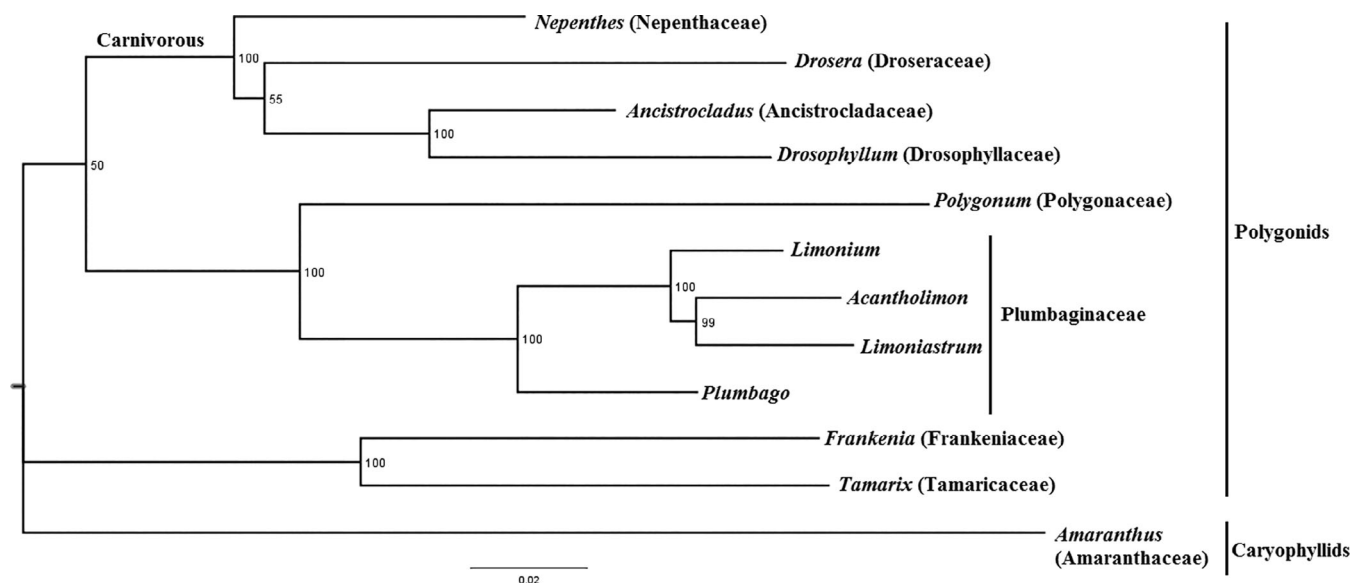


FIGURE 1 Summary of phylogenetic relationships in the Caryophyllales, based on Schäferhoff et al. (2009), using sequence data of the chloroplast *petB* gene (partial), *petB-petD* IGS and *petD* gene (partial) collected in GenBank (<https://www.ncbi.nlm.nih.gov/>; please see Table S1), through Bayesian phylogenetic inference implemented with MrBayes v.3.2.2 (Ronquist et al., 2012), in order to obtain a general phylogenetic tree of Caryophyllales. The resulting tree was visualized in FigTree v1.4, (<http://tree.bio.ed.ac.uk/>). Values to the right of branches are Posterior Probability of MrBayes analysis. The figure shows the major clades of Caryophyllales, separated in two groups where the Caryophyllids clade is exemplified by the Amaranthaceae (used here as an outgroup) and the Polygonids clade is represented by the Ancistrocladaceae, Drosophyllaceae, Droseraceae, Nepenthaceae, Polygonaceae, Plumbaginaceae, Frankeniaceae and Tamaricaceae. Within the Polygonids, there is a clade of non-carnivorous sister taxa, including *Tamarix* and *Frankenia*, and another one with the carnivorous families and another one with the non-carnivorous sister taxa *Polygonum* (Polygonaceae), and *Limonium*, *Acantholimon*, *Limoniastrum* and *Plumbago* (Plumbaginaceae)

uk/affiliates/halophytes/index.php?content=plantStats) so that halophytes represent less than 1% of the approximately 351,000 species of plants whose names have been accepted by taxonomists (<http://www.theplantlist.org/>). In order to avoid any controversy over definitions, we have restricted some of our analyses to plants that tolerate at least 200 mM NaCl (and often seawater) concentrations of salt, plants we call euhalophytes (cf Flowers, Galal, & Bromham, 2010).

Within the Plumbaginaceae, there are nine genera with salt-tolerant species (recorded in eHALOPH: *Aegialitis* R.Br.; *Armeria* (DC.) Willd.; *Ceratostigma* Bunge; *Goniolimon* Boiss.; *Limoniastrum* Heist. ex Fabr.; *Limonium* Mill.; *Myriolimon* Lledó, Erben & M.B.Crespo; *Plumbago* Tourn. ex L. and *Psylliostachys* (Jaub. & Spach) Nevski see Table S2). Both species of *Aegialitis* are salt tolerant trees (mangroves) (Atkinson et al., 1967; Das, 2002) and within the genus *Armeria*, *Armeria maritima* is found in salt marshes (e.g., Rozema, Gude, & Pollack, 1981), on sandy (Köhl, 1997) and on heavy-metal-rich soils (Farago, Mullen, Cole, & Smith, 1980; Heumann, 2002; Neumann, Zurnieden, Lichtenberger, & Leopold, 1995). Just one of the seven species of *Ceratostigma*, *C. plumbaginoides* (Borchert, 1989), and one of the 22 species of *Goniolimon*, *G. tataricum* (Faraday & Thomson, 1986c; Pawlowski, 1963), is a halophyte. Both species of *Limoniastrum* are halophytes (Salama, El-Naggar, & Ramadan, 1999; Zouhaier et al., 2015) as are the two species of *Myriolimon*, *Myriolimon diffusum* (Pount & Revel, 1982) and *M. ferulaceum*, together with one of the 16 species of *Plumbago*, *Plumbago auriculata* (Faraday & Thomson, 1986a; Faraday & Thomson, 1986c) and one of the four species of *Psylliostachys* (*P. spicatus*). The genus *Limonium* has at least 45 halophytic species that have diversified in coastal and inland-saline and gypsum ecosystems (Hernandez-Ledesma et al., 2015; Kubitzki, 1993).

2 | MUCILAGE SECRETING GLANDS

The secretions from plants can be produced from both above and below ground organs for a variety of purposes; secretory structures are found in all angiosperm clades (Brown, George, Neugebauer, & White, 2017), with the potential to influence plant microenvironments in various ways (Galloway, Knox, & Krause, 2020). Some plant species have trichomes (called colleters by Fahn, 1979) that secrete mucilage; examples are seen in young stipules of *Rumex* and *Rheum* (Polygonaceae). The most common colleters have a stalk with at least two rows of cells, side by side (biseriate), supporting several radiate elongated cells (Fahn, 1979).

Within the Plumbaginaceae, mucilage glands (see Figure 2) were described, in the nineteenth century, in *Aegialitis*, *Armeria*, *Ceratostigma*, *Limoniastrum*, *Limonium* and *Plumbago* in comprehensive studies by Wilson (1890) and de Fraine (1916). These mucilage-glands appear in the axils of leaves and on other organs of all the genera, where they are relatively similar in appearance. In some *Limonium* species, these glands occur at the base of the leaf sheath on its upper (adaxial) surface (Batanouny, Hassan, & Fahmy, 1992; de Fraine, 1916; Wilson, 1890; Figure 2a), and secrete large quantities of a transparent colourless, viscous liquid at the base of the petioles. The secreting cells are prismatic,

columnar or conical and radiate from basal collecting cells with straight periclinal walls, without pores in the cuticle envelope covering the gland cells (Batanouny et al., 1992; de Fraine, 1916; Wilson, 1890).

The mucilages may accumulate either at the cell-wall level or in the space between cell wall and protoplast (Trachtenberg & Fahn, 1981) as well as in vacuoles of epidermal cells (Fahn, 1988). A mucilage histochemical test, tannic acid and iron trichloride (Pizzolato & Lillie, 1973), performed on the lower epidermis of *Limonium multiflorum* leaves, demonstrates the presence of mucilage (non-structural polysaccharides) by the appearance of a black colour at the cell-wall level and inside a few epidermal vacuoles (Figure 2b). In *Armeria*, *Ceratostigma* and *Limoniastrum* the mucilage glands are of the same type as in *Limonium*, but in *Armeria* the basal cells are comparatively few in number, but larger than in species of *Limoniastrum* (Wilson, 1890). In *Aegialitis*, the mucilage-secreting cells are found in the axils of the leaves, on laminae, bracts, and sepals, and are very numerous, lying in an oval or circular depression, bounded by regularly-arranged cells (Wilson, 1890). *Plumbago* species have glandular hairs that secrete a sticky mucilage on the petiole and calyx of flowers (Singh, Naidoo, Bharuth, & Baijnath, 2019; Sudhakaran, 2019; Wilson, 1890). It has been hypothesized that sticky exudates function as an aid to pollination by acting as a barrier for insect predators like ants, so preventing predatory attacks on favoured flying insect pollinators (Panicker & Haridasan, 2016). The calyx glands of non-carnivorous *Plumbago* are anatomically similar to the mucilage glands of carnivorous genera *Drosera* and *Drosophyllum*, suggesting a common ancestral gland structure (Thorogood, Bauer, & Hiscock, 2018).

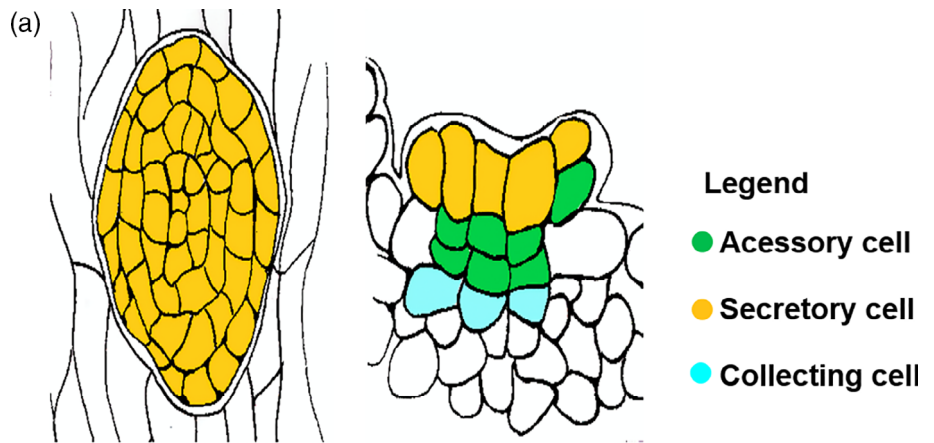
In carnivorous plants, specific multicellular glands are associated with leaves that have been modified to capture prey. Some of the glands producing secretions are supplied with special vascular strands and the surrounding cells show numerous cell wall plasmodesmata (Guo, Yuan, Liu, & Zhu, 2013; Sharifi-Rad et al., 2017), which regulate the transport of substances between adjacent cells. Many internal secretory structures, like glands and ducts of Euphorbiaceae, Papaveraceae, Clusiaceae and Cannabaceae, are associated with vascular bundles, since compound synthesis requires a regular supply of precursors through the phloem. Plastids and photosynthesis itself are known to be involved in the synthetic pathways of many of the compounds secreted (Evans, 2009) and so a localisation near phloem seems to favour the delivery of these different compounds (Sharifi-Rad et al., 2017). However, the presence of vasculature is not an indicator of functional carnivory, since many glands of carnivorous species are not vascularized (e.g., glands of *Nepenthes*; Renner & Specht, 2013). Apart from glands secreting mucilage, other glands have evolved without vascular connections, glands that secrete salt (Tables 1 and 2).

3 | SALT GLANDS

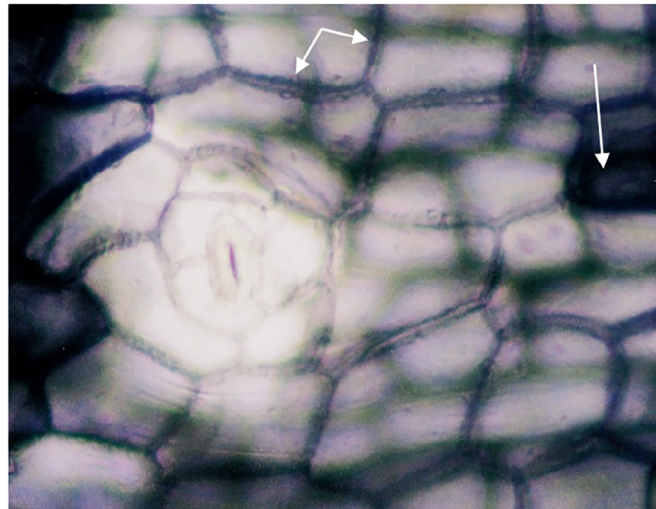
Unlike glands that secrete mucilage, salt-secreting glands are relatively uncommon: they are found in just 12 of the 111 families that contain halophytes. Of the 12 families with salt-secreting halophytes (recretohalophytes), five families contain approximately 90% of the species (Plumbaginaceae, 28%; Poaceae, 21% Amaranthaceae,

FIGURE 2 *Limonium* mucilage glands.

(a). Schematic representation of the cellular organisation of mucilage glands based on de Fraine (1916). Drawing made by Teresa Cardoso Ferreira. (b). Light microscopy of *Limonium multiflorum* lower leaf epidermis. A histochemical test for mucilage (tannic acid and iron trichloride; Pizzolato & Lillie, 1973), reveals a black colour at both the cell wall (small arrows) and vacuoles (large arrow), demonstrating the presence of those non-structural polysaccharides



(b)



20%; Tamaricaceae, 15% and Frankeniaceae, 6%) with seven families containing the remaining 10% (analysis of data in eHALOPH 30/Oct/2019). In all cases, salt glands are epidermal structures, but with anatomical and structural dissimilarities that point to their multiple evolutionary origin (Flowers et al., 2010).

Multicellular salt glands have been described in nine genera of the Plumbaginaceae (Table 1); on leaves and stems, as well as other aerial organs, such as rachis, scapes (inflorescences) and spikes (Salama et al., 1999; Wilson, 1890). Amongst the halophytic species in the family it is likely that all utilize salt glands (see Table 1A), although there are some that thrive in saline habitats/environments, but where the presence of functional glands has yet to be established (e.g., *Myriolimon diffusum*; Table 1). There are also species with glands, whose salt tolerance has yet to be established; for example, *Plumbago zeylanica* (Sudhakaran, 2019) and *P. europaea* (Waisel, 1972) as well as non-halophytes like *Armeria caespitosa* (Giménez-Benavides, Escudero, & Pérez-García, 2005) and *A. canescens* (Scassellati et al., 2016), which have structures similar to salt glands (Table 1B). The presence of structures analogous to salt glands in species where salt tolerance has yet to be established is particularly common in the largest genus within the family, *Limonium* (at least 14 species; see Table 2A,B; there are 34 halophytic species of *Limonium* with salt glands).

The salt glands of the Plumbaginaceae are complex, consisting of up to 40 cells. Within most species, the glands are composed of 16 cells (Thomson et al., 1988); only the glands of *Limonium guyanianum* (with 32 cells; Tables 1 and 2) and those of *Aegialitis* (24 or 40 cells) have more. Multicellular salt glands have also been described in the noncore Caryophyllales families Frankeniaceae and Tamaricaceae (Dassanayake & Larkin, 2017; Fahh, 1988; Flowers et al., 2010; Grigore & Toma, 2017; Thomson et al., 1988) - in four genera and 58 species (Table 3; Table S4). Although the number of halophytes within the Frankeniaceae and Tamaricaceae is small, just 2 and 1%, respectively, of all halophytes, 80% of salt-tolerant species within the Frankeniaceae and 67% of salt-tolerant species within the Tamaricaceae have been recorded as having salt glands (eHALOPH, October 2019). Glands within the Frankeniaceae and Tamaricaceae have fewer cells, than genera in the Plumbaginaceae - generally eight (Table S4), rather than 16 in the species within the Plumbaginaceae (Tables 1, 2 and 3). This smaller number of cells per gland (8) is associated with a higher frequency (median of 31 per mm²; $n = 14$ within the Frankeniaceae and Tamaricaceae, Table S4) than seen in the Plumbaginaceae (median of 12 per mm², $n = 22$, Tables 1, and S4). Notably, in members of the Polygonaceae, sister group of Plumbaginaceae, no species is recorded as having salt glands.

TABLE 1 Genera and species within the Plumbaginaceae (from Plants Of the World Online, POWO) reported to have salt glands (from eHALOPH), their salt tolerance as indicated by inclusion in the database eHALOPH, the position of the glands on the leaf, their frequency and cellular makeup, together with the main elements secreted and rates of efflux

Genus and species	Species with salt glands ^a	Gland structure	Number / mm ²	Number of cells	Main elements secreted and efflux	Na/Cl ratio	Reference & notes
A species in eHALOPH							
<i>Aegialitis</i> R.Br.	2						
<i>annulata</i>	✓	3 rings 8 cells	9	24	Cl 11–62 pmol/cm ² /s	0.79	Atkinson et al., 1967
<i>annulata</i>	✓	8 rings 5 cells		40			Faraday & Thomson, 1986c
<i>annulata</i>	✓				Na, Cl, K, Ca	1.1	Faraday & Thomson, 1986a
<i>rotundifolia</i>	✓						Seshavatharam & Srivalli, 1989; Yuan, Chen, Leng, & Wang, 2013
<i>rotundifolia</i>	✓	8 cells per ring	?				Das, 2002
<i>rotundifolia</i>	✓			24			Wang B-S, Unpublished data
<i>Armeria</i> (DC.) Willd.	1						
<i>maritima</i>	✓				HCO ₃ , Cl, Na, K, Ca, Mg	0.96	Baumeister & Ziffus, 1981
<i>maritima</i>	✓		6		Na, K, Ca: 0.7 pmol/gland/h		Rozema et al., 1981
<i>maritima</i>	✓	4 quadrants		16	NaCl		Ruhland, 1915 (synonym <i>A. vulgaris</i>)
<i>Ceratostigma</i> Bunge	1						
<i>plumbaginoides</i>	✓				Ca		Borchert, 1989
<i>plumbaginoides</i>	✓	4 quadrants		16	Mg, Ca		Balsamo & Thomson, 1996; Faraday & Thomson, 1986a; Faraday & Thomson, 1986c
<i>Goniolimon</i> Boiss.	1						
<i>tataricum</i>	✓	4 quadrants	31	16			Faraday & Thomson, 1986c (misspelled as <i>tatericum</i>); Waisel, 1972
<i>Limoniastrum</i> Heist. Ex Fabr.	2						
<i>guyonianum</i>	✓			32	Mg & Ca or Na & Cl		Zouhaier et al., 2015; Mg & Ca when no NaCl in soil
<i>monopetalum</i>	✓			16	Na, Cl	0.69	Salama et al., 1999
<i>monopetalum</i>	✓				K 0.6–2.1 μmol/g fw		Ramadan, 1997
<i>monopetalum</i>	✓	12 + 4 cells	22	16			Batanouny & Abo, 1977
<i>monopetalum</i>	✓		12		CaCO ₃ , NaCl		Batanouny & Abo, 1977
<i>Limonium</i> Mill.	40						
See Table 2							
<i>Myriolimon</i> Liedó, Erben & M.B.Crespo	2						
<i>diffusum</i>	?						
<i>ferulaceum</i>	?						

TABLE 1 (Continued)

Genus and species	Species with salt glands ^a	Gland structure	Number / mm ²	Number of cells	Main elements secreted and efflux	Na/Cl ratio	Reference & notes
<i>Plumbago</i> Tourn. Ex L.	1						
<i>auriculata</i>	✓			16?	Mg, Ca		Faraday & Thomson, 1986a; Faraday & Thomson, 1986c
<i>auriculata</i>	✓		25				Waisel, 1972
<i>Psyllostachys</i> (Jaub. & Spach) Nevski	1						
<i>spicatus</i>	?						
B species NOT in eHALOPH							
<i>Acantholimon</i> Boiss							
<i>androsaceum</i>	✓	8 cells + 4 subsidiary cells		12			Bokhari, 1971
<i>glumaceum</i>	✓	8 cells + 4 subsidiary cells		12			Bokhari, 1971
<i>lycopodioides</i>	✓	8 cells + 4 subsidiary cells		12			Bokhari, 1971
<i>Armeria</i> (DC.) Willd.							
<i>maritima</i> ssp. <i>halleri</i>	✓				K, Ca, Cu, Zn		Neumann et al., 1995
<i>maritima</i> ssp. <i>halleri</i>	✓				S, Zn		Heumann, 2002
<i>canescens</i>	✓	4 quadrants	2–16	16			Scassellati, Pasqua, Valletta, & Abbate, 2016
<i>Plumbago</i> Tourn. Ex L.							
<i>zeylanica</i>	✓			8			Sudhakaran, 2019

Note: Representatives of the genus *Limonium* are presented separately, in Table 2.

^aThe presence of glands is recorded with a ✓ in column 2; a ? indicates the presence of functional salt glands is uncertain or not known. Entries in bold text relate to genera, rather than individual species.

TABLE 2 Glandular attributes for species of *Limonium* (A), reported to be salt tolerant (are included in eHALOPH) and (B) without proven salt tolerance

Species (synonym in parentheses)	Gland structure	Number of glands/mm ²	Number of cells	Main elements secreted and efflux	Reference
A <i>Limonium</i> species that are salt tolerant - in eHALOPH					
<i>aureum</i>	12 + 4 collecting cells		12		Ni, Tan, & Shen, 2012
<i>axillare</i>	4 rings + 4 basal cells		16		Salama et al., 1999
<i>axillare</i>	Sunken				Akhani, Malekmohammadi, Mahdavi, Gharibiyani, & Chase, 2013
<i>bellidifolium</i>	8 gland cells + 4 subsidiary cells	9	12		de Fraigne, 1916
<i>bicolor</i>	4 each of outer cup cells, inner cup cells, accessory cells and secretory cells		16	Na, K, Mg, Ca, Cl, SO ₄ ²⁻ , NO ₃ ⁻ , Na: 40–225 pmol/gland/h, Cl: 48–226 pmol/gland/h	Feng et al., 2014
<i>bicolor</i>		12–16		Na: 110 pmol/gland/h	Leng, Yuan, Dong, Wang, & Wang, 2018
<i>binervosum</i>	Not recorded	10			de Fraigne, 1916; Grigore & Toma, 2010;
<i>brasiliense</i>	4 secretory cells, 4 collecting cells and remaining accessory cells		16		Bastos, Perazzolo, & Gorgen, 1993
<i>brasiliense</i>	12 + 5 to 8 cells				Gancedo, de Medeiros, Milaneze-Gutierrez, & de Mello, 2018
<i>californicum</i>	4 rings		16		Faraday & Thomson, 1986c
<i>caspium</i>	Present but no detail				Akhani et al., 2013
<i>delicatulum</i>	12 cells in 3 rings + 4 collecting cells	20–29	16		Batanouny et al., 1992; Blazquez, 1985
<i>franchetii</i>	4 each of secreting cells, internal and external "goblet" cells collecting cells	8	20?		Xin, Tan, & Chu, 2012
<i>girardianum</i>	Present but no detail				Al Hassan et al., 2017
<i>gmelinii</i>	4 rings	7	16		Faraday & Thomson, 1986c; Ruhland, 1915
<i>gmelinii</i>	At surface				Akhani et al., 2013
<i>gmelinii</i>		12			Zhou, Liu, & Wang, 2007
<i>gmelinii</i>		10–12		Na: 290 pmol/gland/h	Leng et al., 2018
<i>gmelinii</i>	Quadrant		16		Ruhland, 1915
<i>gmelinii</i>		6–8			Daraban, Mihali, Turcus, Ardelean, & Arsene, 2013
<i>gmelinii</i>		11			Zorić, Anačkov, Karanović, & Luković, 2013
<i>graecum</i>	Not recorded	10			Waisel, 1972
<i>iranicum</i>	Deeply sunken				Akhani et al., 2013
<i>llanceolatum</i>			16		Caperta, Unpublished data
<i>lobatum</i>	At surface				Akhani et al., 2013
<i>multiflorum</i>	At surface		16		This study
<i>narbonense</i>	At surface		16		Al Hassan et al., 2017 This study
<i>nudum</i>	At surface				Akhani et al., 2013
<i>nydeggeri</i>	At surface		16		This study
<i>oleifolium</i> (syn. = <i>virgatum</i>)	Present but no detail				Andres, 1989; Colombo, 2002

TABLE 2 (Continued)

Species (synonym in parentheses)	Gland structure	Number of glands/mm ²	Number of cells	Main elements secreted and efflux	Reference
<i>otolepis</i>		9			Zhou et al., 2007
<i>otolepis</i>	At surface	12			Akhani et al., 2013
<i>ovalifolium</i>	Present but no detail				Denaeyer-De Smet, 1970
<i>pectinatum</i>			16	Na: 0.2 nmol/gfw/s	Jung & Luttge, 1980; Morales, Olmos, Torrecillas, Sanchez-Blanco, & Alarcon, 2001
<i>perezii</i>	4 quadrants		16		Faraday & Thomson, 1986c
<i>perezii</i>				Na, K, Ca, Cl: 20–410 pmol/gland/h	Faraday & Thomson, 1986b
<i>platyphyllum</i>	Cross sections				Vassilyev & Stepanova, 1990
<i>pruinatum</i>	16 secretory cells + 4 collecting cells	16	16		Salama et al., 1999; Waisel, 1972
<i>reniforme</i>	At surface	12–16			Akhani et al., 2013
<i>santapolense</i>	Present but no detail				Al Hassan et al., 2017
<i>sinense</i>	16 + 4 collecting cells		20		Xin, Tan, & Chu, 2011
<i>sinuatum</i>	4 quadrants	10	16		Faraday & Thomson, 1986c; Salama et al., 1999; Waisel, 1972
<i>stocksii</i>	Sunken				Akhani et al., 2013
<i>suffruticosum</i>	Deeply sunken				Akhani et al., 2013
<i>vulgare</i>	16 cells in 4 circles with 4 basal cells		20		Faraday & Thomson, 1986c; Salama et al., 1999;
<i>vulgare</i>		30			Rozema et al., 1981
B Limonium species that are not in eHALOPH					
<i>aegusae</i>			16		Colombo, 2002
<i>albidum</i>			16		Colombo, 2002; Colombo & Trapani, 1992
<i>bocconeii</i>			12		Colombo, 2002
<i>carosum</i>	Sunken				Akhani et al., 2013
<i>intermedium</i>			16		Colombo & Trapani, 1992
<i>dictyophorum</i> (syn. <i>L. anfractum</i>)		7			Zorić et al., 2013
<i>lojaconi</i>			12		Colombo, 2002
<i>lopadusanum</i>			16		Colombo & Trapani, 1992
<i>palmyrense</i>	Deeply sunken				Weiglin & Winter, 1991
<i>perfoliatum</i>	At surface				Akhani et al., 2013
<i>pignantii</i>			12		Colombo, 2002
<i>ponzoi</i>			16		Colombo, 2002
<i>serpenticum</i>	Present but no detail				Pino Perez, Javier Silva-Pando, & Pino Perez, 2016
<i>sogdianum</i>	At surface				Akhani et al., 2013

3.1 | Morphological studies of salt glands

Ruhland (1915) using cross and longitudinal sections of leaves of *Statice gmelinii* (syn. *Limonium gmelinii*) illustrated a detailed anatomical description of the complex 16-celled salt glands (with cells arranged

in four quadrants; Figure 3) found within the Plumbaginaceae (see Grigore and Toma, 2016 and Grigore & Toma, 2017 for a history of the description of these glandular structures). Ruhland (1915) also reported this cellular arrangement in *Statice incana* (synonym: *Goniolimon incanum*), *S. latifolia* (synonym: *Limonium platyphyllum*) and

TABLE 3 Genera and species within the Tamaricaceae and Frankeniaceae (from Plants of the World Online, POWO) reported (from eHALOPH) to have salt glands

Genus and species (synonym in parentheses)	Number of species POWO	Species In eHALOPH	Species with glands
Frankeniaceae	78	10	11
<i>Frankenia</i> L.	78	10	11
Tamaricaceae	112	40	29
<i>Myricaria</i> Desv.	14	0	3
<i>Reaumuria</i> L.	25	5	3
<i>Tamarix</i> L.	72	29	23

Note: For more detail see Table S4.

in *A. maritima* (also then known as *A. vulgaris*). Indeed in the Plumbaginaceae this organisation is well preserved, with the glands of most species within the family consisting of 16 cells (Table 1, 2; Balsamo & Thomson, 1993; Faraday & Thomson, 1986c; Salama et al., 1999; Thomson et al., 1988), characteristically differentiated into secretory, collecting and accessory cells, in a cuticle-lined structure (Figure 3a). There is, however, some disparity in the literature as to whether or not four large basal cells are included in the gland.¹

In *Armeria*, *Ceratostigma* and *Goniolimon* the 16 cells of the salt glands are arranged in four quadrants. The four central secretory cells are surrounded by accessory cells, both being enclosed by two other layers, each of them consisting of four cells (Fahn, 1988; Faraday & Thomson, 1986c; Feng et al., 2014; Salama et al., 1999). In leaves of *Aegialitis annulata*, salt glands appear to be organized into eight rings of five cells (Faraday & Thomson, 1986c) whereas in *A. rotundifolia* they are present within a cup-shaped crypt in the epidermal layer (Das, 2002) and consist of rings of eight cells (Atkinson et al., 1967). In *L. guyonianum* leaves, however, each salt gland comprises 32 cells, 16 in cross section (Figure 3b), including two huge collecting cells, six accessory cells, and a central part representing the secretory structure containing eight cells - four collecting cells, each one surmounted by an apical cell (Zouhaier et al., 2015). Although the study by Faraday and Thomson (1986c) reports 16 cells for *P. auriculata* and that of Sudhakaran (2019) of *P. zeylanica* shows eight cells, it is not possible to confirm either number from the published material.

In *Limonium* species a complex 16-celled salt-gland structure is commonly reported (Table 2; Figure 3a; Batanouny et al., 1992; Faraday & Thomson, 1986c; Salama et al., 1999; Vassilyev & Stepanova, 1990; Yuan et al., 2016; Ziegler & Lüttge, 1966), although other arrangements of cells have also been found. For instance, in *L. aureum*, *L. bocconeii*, *L. lojaconi* and in *L. pignattii* the mature gland seems to include 12 cells (four central secretory, adjacent and collecting cells) (Ni et al., 2012). In *L. sinense* and *L. franchetii* the mature salt gland is described as a complex structure with 20 cells - four secreting cells each accompanied by an adjacent cell and bounded by four internal and four accessory cells, and four collecting cells (Xin et al., 2011, 2012). As in previous cases, however, it is possible that the number of cells within a gland is open to different interpretations. Here we show that *L. multiflorum*,

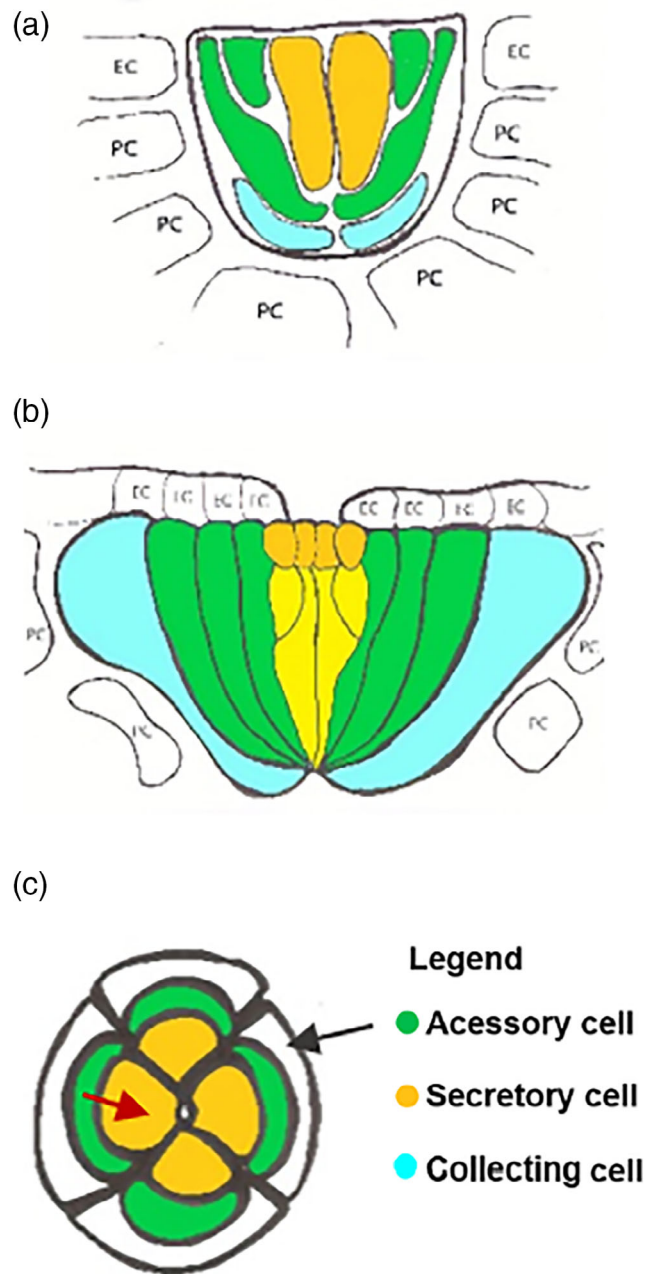


FIGURE 3 Schematic representation of the cellular organisation of the salt gland structures found in Plumbaginaceae. From left to right: diagrams are based on images of *Limonium bicolor* (Ruhland, 1915; Yuan, Leng, & Wang, 2016) (3a); *Limoniastrum guyonianum* (Zouhaier et al., 2015) (3b) and *Plumbago zeylanica* (Sudhakaran, 2019) (3c). In Figure 3 (c) the collecting cells are below the epidermis and not seen in the surface view. The distinct components of the glands are represented by secretory (yellow), collecting (blue) and accessory (green) cells. Red arrow secretory cell; black arrow epidermal cell. Acronyms EC – Epidermal Cell and PC – Parenchyma Cell. Drawing made by Teresa Cardoso Ferreira

L. narbonense *L. nydeggeri* and *Limonium ovalifolium* all present salt glands with 16 cells, arranged in quadrants (Figure 4), where cells have a spatial and functional differentiation. In each quadrant, there are four cells: a

basal collecting cell and two accessory cells, and these surround a secretory cell (Figure 4a).

The glandular complex can vary in its position in the epidermis. In leaves, the glands can be seen on the surface at the level of the other epidermal cells or deeply sunken in the leaves, being then side by side with the mesophyll cells (Tables 1 and 2, Figures 4a,b; Akhani et al., 2013; Salama et al., 1999; Thomson et al., 1988). In stems, scapes and spikelets, the insertion of glands is similar to that in the leaves: glands may protrude from the surface as observed in *A. maritima* (Bernard & Lefebvre, 2001) or even be located on the top of a special elevated cortical structure as reported in *Limonium pruinosum* (Salama et al., 1999). A dense and thick cuticle is present around the whole group of cells forming the gland, which is confirmed by fluorescence microscopy on cross sections of *L. multiflorum* leaves (Figure 4b) and on surface view of *L. narbonense* leaves (Figure 4c). This seems to be fundamental in protecting epidermal and mesophyll cells from salt damage (Thomson, Berry, & Liu, 1969). The four secretory cells excrete the mineral solutions through four visible pores (one pore per cell) (Figure 4d), which gives a peculiar appearance to the epidermis, whose cuticle can have different types of wax deposits (Figure 4e,f). The glands have no direct connection with the vascular bundles (Fahn, 1988).

In summary, reported differences in cell number between 12 and 20 cells appear to us to be an arbitrary difference of interpretation of which cells are included in the gland. We interpret the data as showing most members of the Plumbaginaceae have 16-celled glands; the exceptions are *A. annulata*, *A. rotundifolia* and *L. guyanianum*. Species within the Frankeniaceae and Tamaricaceae have eight-celled glands with each being comprised of six secreting cells and two collecting cells totalling eight cells (Table S4).

3.2 | Physiology of salt secretion

In the late nineteenth century, the secretion of CaCO_3 by glands on the surface of leaves of *Armeria*, *Statice*, *Goniolimon*, *Limoniastrium* and *Plumbago* was demonstrated (Braconnot, 1836). The organs responsible were drawn by Mettenius (1856) and Licopoli (1867) and called "Licopoli" (Maury, 1886), "Mettenius" (Wilson, 1890) or even "calciferous" glands (Licopoli, 1870). Their illustrations are of structures similar to those we now call salt glands (compare Grigore & Toma, 2017 and Wilson, 1890) and indeed Sakai (1974) suggested that "in all probability chalk glands and salt glands are not separate secretory structures" based on the low ion selectivity shown by the way that *Tamarix aphylla* can predominantly secrete either Na or Ca depending on substrate. Advances in analytical techniques, particularly analytical x-ray microscopy, have revealed the range of elements that are secreted by glands (Faraday & Thomson, 1986a; Feng et al., 2014; Salama et al., 1999). While the secretions from many species do contain Ca and Mg (Table 1), the glands are capable of secreting a variety of ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Zn^{2+} , Cl^- , HCO_3^- , SO_4^{2-} ; see also Thomson et al., 1988) with fluxes of Na^+ and Cl^- reaching values of over 200 pmol/gland/h (Table 2). Crystals on the surface of leaves of

A. maritima growing on contaminated soil contained Cu and lesser amounts of Zn, Ni, Fe and Mn (Neumann et al., 1995). In plants in solution culture, Heumann (2002) identified Zn within cells of the salt glands. Salts on the surface of leaves of *Tamarix africana* growing on a contaminated salt marsh contained As, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn (Santos et al., 2017), while plants of *T. smyrnensis* growing in solution culture have been shown to secrete Pb and Cd (Kadukova, Manousaki, & Kalogerakis, 2008). Nevertheless, Na and Cl dominate the secretions of plants growing on maritime saltmarshes.

The presence of salt glands in some, but not all, halophytes raises a question of how some species are able to tolerate salinity without the ability to excrete salt while others utilize glands. Are these glands required for salt tolerance in some species or are they simply a structure that evolved for other reasons and have become adapted to secrete salts when present? Here we examine the case for the more extreme halophytes, the euhalophytes - these are plants that can tolerate at least 200 mM salt and so are amongst the most salt-tolerant species of plants. There are 524 euhalophytes listed in eHALOPH, of which just 74 species are reported to have salt glands - with the Amaranthaceae, Poaceae and Plumbaginaceae accounting for 80% of these species. The Plumbaginaceae is, however, remarkable in that most (86%) of its euhalophytes have salt glands as compared to 31% of the Poaceae and 18% in the Amaranthaceae. In the related Frankeniaceae and Tamaricaceae, the proportion of euhalophytic species with salt glands is also remarkably high (Frankeniaceae, 100% and Tamaricaceae, 60%). So, why the importance of salt glands? The salt glands of the Amaranthaceae and Poaceae are bicellular and so different from the complex glands seen in the other families. Those of the Amaranthaceae are external bladders (Osmond, Lüttge, West, Pallaghy, & Shacher-Hill, 1969; Shabala, Bose, & Hedrich, 2014) and can be interpreted as a salt-storage system, analogous to the storage cells seen in the succulent leaves in other members of the family. Such bladders are not essential for extreme salt tolerance in the Amaranthaceae; they are absent in genera such as *Salicornia*, *Suaeda* or *Tecticornia* and, where present, do not excrete ions in the way that the glands of the Poaceae do. The microhairs of the Poaceae (present in all but the Pooideae) do secrete salt (see Dassanayake & Larkin, 2017), but again are clearly not essential for tolerance of high salinities in the Poaceae, since there are, euhalophytes in, for example, the genera *Paspalum*, *Phragmites* and *Puccinellia* that do not have salt glands. So, why are salt glands such a common feature of euhalophytic members of the Plumbaginaceae, Frankeniaceae and Tamaricaceae? Is it that species in these three families are less efficient than the members of the Amaranthaceae in limiting the access of ions to the xylem and so have to excrete salt that reaches the leaves?

To survive salinity, plants have to balance the delivery ions with their growth rate. It is clear that all plants are able to restrict the entry of ions reaching the xylem vessels to some extent (Munns & Tester, 2008) - so called exclusion. Salt-sensitive species, such as rice, appear to be rather poor at this process and rice has been shown to have a "bypass" flow; a leak of ions through areas of the root system that have a poorly developed endodermis (Faiyue, Al-Azzawi, & Flowers, 2010; Yeo, Yeo, & Flowers, 1987). Estimates of "exclusion"

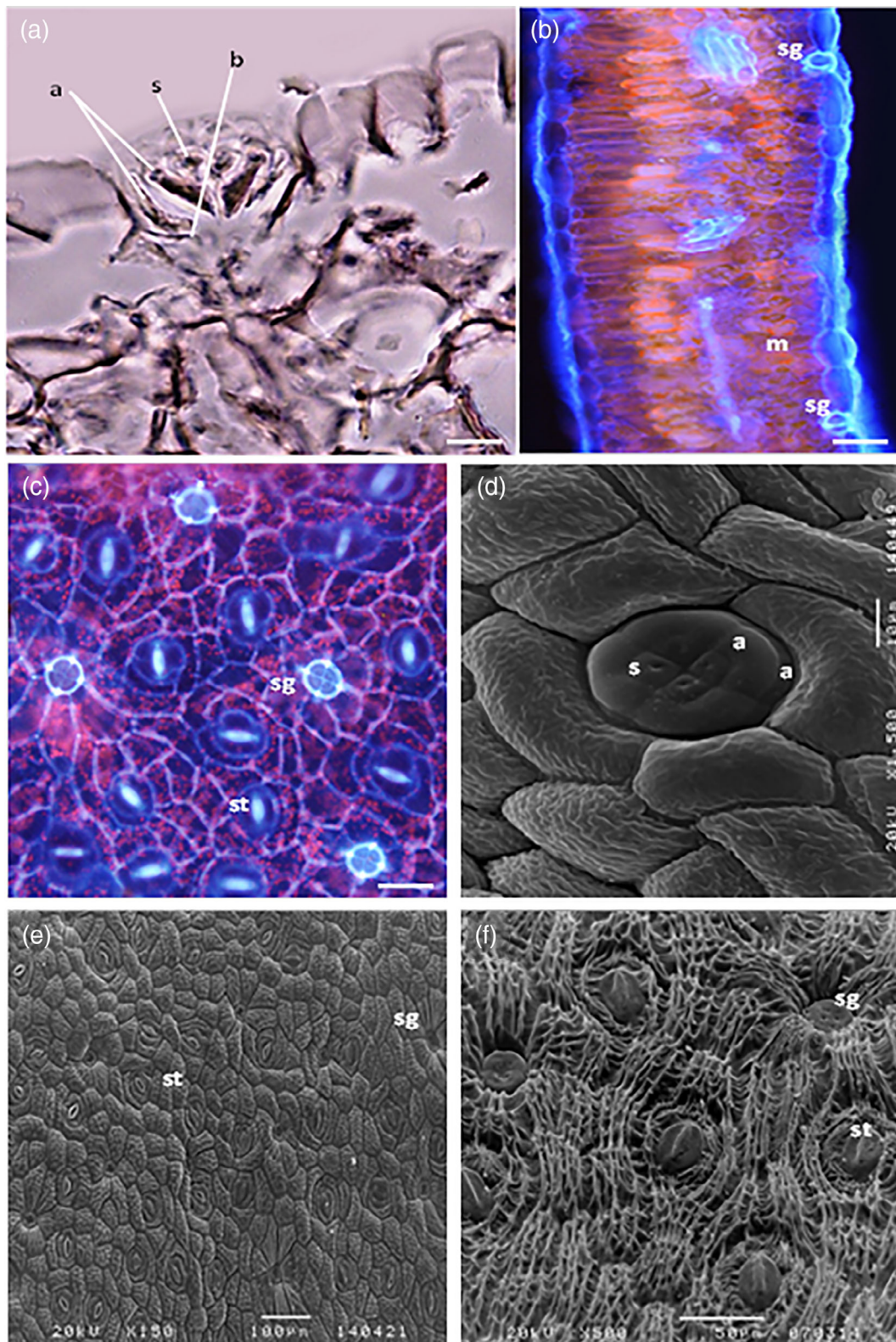


FIGURE 4 Legend on next page.

can be made by comparing ion concentrations in the xylem sap to those in the medium. Neither parameter is particularly easy to determine: medium concentrations are only easy if plants are growing in a liquid medium while xylem concentrations have to be estimated from

change in shoot content over time (hours to weeks) divided by the volume of water transpired in that time. Nevertheless, exclusion values for halophytes have been calculated and range from 77 to 99% (Flowers, 1985; Reef & Lovelock, 2015). The apparent “low exclusion”

can be accounted for by the fact that many halophytes utilize Na and Cl in growth so that xylem concentrations of Na⁺ can be high (see, e.g., Yeo & Flowers, 1986). However, once the delivery of Na⁺ and Cl⁻ exceeds the capacity for the plant to compartmentalize those ions, then growth is reduced. This situation might be avoided or mitigated if excess ions are excreted, but there is too little evidence to allow a clear conclusion. Rozema et al. (1981) showed that excretion by glands can be an important aspect of balancing the salt load in shoots of some species. For plants growing in 200 mM NaCl for 3 days, they (Rozema et al., 1981) calculated excretion as a proportion of increase in content for *Spartina anglica* to be 1, for *Limonium vulgare*, the ratio was 0.3; for *Glaux maritima*, 0.1 and for *A. maritima*, 0.04. It is clear that the two members of the Plumbaginaceae (*Limonium* and *Armeria*) were not particularly reliant on secretion, although even a small proportion could be critical for survival. In a study of mangroves, Reef and Lovelock (2015) concluded that "... salt excretion (the salt gland trait) is not sufficient or necessary to confer high levels of salinity tolerance. The presence of salt glands ... is also not linked to levels of salt exclusion." Unfortunately, there is no systematic data for halophytes that compares secretion as a proportion of uptake or the xylem concentrations of Na⁺ and/or Cl⁻ that would allow us to evaluate whether or not glands are required for salt tolerance in some species.

As salt tolerant species in the Plumbaginaceae appear to utilize salt glands and yet there is a significant number of species with glands that are not halophytes (Tables 2B, 3, and below) we have looked for alternative explanations for the presence of glands in members of the family and of species within the Frankeniaceae and Tamaricaceae. One possibility is the regulation of Ca concentrations. Ca is an important determinant of the response of plants to salinity, not only in its role in signalling, but also through its effects on cell walls and membranes (Greenway & Munns, 1980; Hadi & Karimi, 2012). Ca is essential for plant growth (White & Broadley, 2003) and yet the concentration of cytoplasmic free Ca²⁺ is low (sub-micromolar, Broadley et al., 2003; Tang & Luan, 2017), reflecting its role as an important signalling molecule. Since there is a strong inward driving force for Ca²⁺ into cells (Demidchik, Shabala, Isayenkov, Cuin, & Pottosin, 2018), plants use a variety of means to regulate their Ca concentrations (Tang & Luan, 2017), amongst which is the ability to precipitate calcium oxalate (Franceschi & Nakata, 2005), seen particularly in the Amaranthaceae and Polygonaceae within the Caryophyllales (White & Broadley, 2003). As far as we are aware, however, little is known of the Ca relations of the Plumbaginaceae,

Frankeniaceae, or Tamaricaceae, although shoot Ca concentrations in the Plumbaginaceae and Tamaricaceae, appear to be in the lower range of values seen across plants (Broadley et al., 2003). The range of Ca concentrations seen in plants is 0.11 to 4.41% of shoot dry weight as calculated by Broadley et al. (2003) from data in the literature. The value for the shoots of *Tamarix ramosissima* is in the middle of this range, at 1.97% of the dry weight. In a hydroponic experiment they (Broadley et al., 2003) recorded the shoot Ca of *A. maritima* to be 0.59%. Since Ca is secreted from the glands of both *T. ramosissima* and *A. maritima* (Tables 2 and S4), this is a means by which shoot Ca concentrations could be regulated. We hypothesize that multicellular salt glands could have evolved in the Plumbaginaceae, Frankeniaceae and Tamaricaceae to regulate shoot Ca concentrations and perhaps the balance between Ca and Mg (Tang & Luan, 2017). Over the course of time, this allowed species of these families to colonize drier saline soils as well as seawater (*Aegilitis*; Table 1).

3.3 | Evolution of salt glands

The Plumbaginaceae is well-known for salt and drought tolerance, with genera and species adapted to arid environments and a wide range of saline habitats – adaptations involving anatomy (e.g., multicellular glands) and physiology (e.g., osmoprotective compounds; Slama, Abdelly, Bouchereau, Flowers, & Savouré, 2015). In the Plumbaginaceae, salt glands occur in species that can grow in tidal areas (including the mangrove *Aegilitis*; Das, Mishra, & Mohanty, 2006) and in higher drier parts of salt marshes subjected to high salinity (e.g., *Limonium* and *Limoniastrum*; Álvarez & Manzanares, 2017; Costa et al., 2014; Dawson & Ingrouille, 1995; de Fraine, 1916; Zhao, Song, Feng, Zhao, & Liu, 2011). Glands are also present in genera that grow on rocky coasts in incipient soils exposed to deposition of airborne salt spray (as in *Limonium*, "rocky species"; Caperta et al., 2014) and in *Ameria* species of coastal sand dunes (Arseni & Diez-Garretas, 2017). At least one *Goniolimon* species that thrives in steppe-like habitats (xerophilous pastures and rocky grounds) in hilly regions has salt glands on its leaves and stems (Buzurovic, Stevanovic, Niketic, Jakovljevic, & Tomovic, 2013; Faraday & Thomson, 1986c; Waisel, 1972), although glands are not present in *Acantholimon* that colonised mountainous regions in dry habitats on gravelly and stony soils or on exposed rocks (Moharrek et al., 2019). The gland character is found in species ranging from perennial herbs

FIGURE 4 Salt glands in leaves of *Limonium* species: **a**, light microscopy; **b** and **c**, fluorescence microscopy; **d**, **e** and **f**, scanning electronic microscopy (plant material fixed in a 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.2, for 5 h at 4 °C following Hayat (1981). The material was dehydrated in a graded ethanol series (30, 50, 75 to 100% ethanol for 30 min each). Then, leaves were dried on a Critical Point Polaron BioRad E3500 and coated with a thin layer of gold on a Jeol JFC-1200. Observations were carried out at 15 kV on a JSM-5220 LV scanning electron microscope equipped with a direct image acquisition system). (a) *Limonium narbonense* paraffin leaf cross section cut on a Minot microtome as described in and Ruzin (1999); detail of a salt gland, with a typical 16 cell arrangement (in cross section 8 cells are visible; scale bar 10 μm). (b) *Limonium multiflorum* hand leaf cross section, autofluorescence, under UV light, (scale bar 50 μm). (c) *Limonium narbonense* leaf abaxial surface, autofluorescence, under UV light, (scale bar 37 μm). (d) *Limonium nydeggeri* leaf abaxial surface, salt gland detail, with 4 secretory cells each one with its own secretion pore, surrounded by 8 accessory cells (4 completely visible). (e) *Limonium nydeggeri* abaxial surface. (f) *Limonium multiflorum* abaxial surface. Acronyms: sg - salt gland; b - collecting basal cells; a - accessory cells; s - secretory cells; st - stomata; and m - mesophyll cells

with slightly fleshy leaves as in *Limonium*, to cushion-forming dwarf shrubs as in *Armeria*, coastal shrubs as in *Limoniasstrum* and small trees as in *Aegialitis* (Table 2), suggesting a plesiomorphic origin of this halophytic trait. Genetic studies in *A. maritima* provide strong evidence that metalicolous populations have been derived from the ancestral non-metallicolous populations repeatedly and independently in different geographical regions (Baumbach & Hellwig, 2007).

The evolutionary pathways leading to the “salt glands syndrome” are not well understood. Our phylogenetic reconstruction revealed several main findings. First, salt glands are absent in salt-tolerant members of the Polygonaceae. Second, the most recent common ancestor of the family Plumbaginaceae likely possessed salt glands without vasculature; such glands are also present in the related non-carnivorous families, Frankeniaceae, and Tamaricaceae. Within the Plumbaginaceae, the subfamily *Limonioideae* presents more genera with salt glands and with a greater variety of structures than the subfamily *Plumbaginoideae*: the *Limonioideae* appear to have diversified more and more recently than the *Plumbaginoideae*. In the related carnivorous families (Figure 5), the multicellular glands can be sessile, stalked, or pitted, and may contain xylem and phloem; they have evolved a variety of different functions such as lures, generating trapping glue, providing a drowning mechanism and a digestive (enzyme) medium as well as the absorption of water vapour at night (Juniper, Robins, & Joel, 1989; Renner & Specht, 2013).

Notwithstanding the fact that salt glands are shared by members of Plumbaginaceae and related Tamaricaceae and Frankeniaceae, the divergence date of these families is difficult to estimate since there are no recorded reliable macrofossils of these families. Although there are microfossils from the Plumbaginaceae in the form of pollen grains, those of *Acantholimon*, *Armeria*, *Goniolimon*, *Limonium* and *Psylliostachys* are difficult to distinguish and are referred to as *Limonium* or *Armeria* type (Baker, 1948, 1953; Skvarla & Nowicke, 1976; Weber-El Ghobary, 1984). Hence, estimating a divergence date using fossil pollen is not feasible. Based on molecular dating, it has been estimated that the split between the Polygonaceae and its sister group the Plumbaginaceae is relatively ancient, occurring about 90.7–125.0 Ma (million years ago, Schuster, Setaro, & Kron, 2013). Bell, Soltis, and Soltis (2010) dated minimum and maximum ages for the Tamaricaceae at 60–58 Ma compared to the age of Plumbaginaceae at 27–57 Ma. Molecular analyses placed the split between *Plumbago* and *Limonium* between 18–16 Ma during the Miocene (Lledó et al., 2005), whereas the non-halophyte *Acantholimon sensu lato* diverged from its sister genus about 7.5 Ma, beginning between the Late Miocene and the Pliocene (Moharrek et al., 2019).

In the current revision, a Bayesian inference analysis of the Plumbaginaceae using chloroplast tRNA-Leu (*trnL*) gene and *trnL-trnF* intergenic spacer, shows that genera within the Plumbaginaceae are separated from sister salt-tolerant members of the Polygonaceae (*Polygonum*) (PP = 1) and of Tamaricaceae (*Tamarix*), here used as outgroups (Figure 3; Table S3). Within the Plumbaginaceae, two major clades represent the two monophyletic subfamilies *Plumbaginoideae* and *Limonioideae* (PP = 1). Inside the *Plumbaginoideae*, *Plumbago*, a genus with halophytes with salt glands, is close to *Dyerophytum*, and

sister of the *Ceratostigma* clade, which again has halophytes; together they form a clade with the monospecific *Plumbagella*. In the subfamily *Limonioideae*, the genus *Limonium*, with many halophytes and whose members present salt glands, seems to be the oldest genus within the subfamily. *Armeria*, with a halophytic species with glands and non-halophytic species also with glands form a clade together with the halophyte *Psylliostachys*. Another clade with halophytes with salt glands is that grouping *Limoniasstrum*, sister to *Ceratolimon*, and close to *Myriolimon* and to *Ikonnikovia*. A clade with non-halophytes with PP = 1 is formed by *Acantholimon*, *Dictyolimon*, *Bukiniczia*, *Chaetolimon*, *Vassilczenkoa*, *Popoviolimon* and *Cephalorhizum*. To sum up, we might presume that the establishment of lineages with salt glands took place upon the split of between the Polygonaceae and its sister group Plumbaginaceae, but how it is that this separation took place is unclear.

Limonium species seemed to diversify at a fast pace during the Messinian (Lledó et al., 2005; this study), when the climatic conditions were drier than in previous ages and plants tolerated low water potentials (Valiente-Banuet, Rumebe, Verdu, & Callaway, 2006). This might have facilitated the diversification of species possessing physiological and anatomical adaptations that allowed colonisation of harsh and arid conditions; for example, halophytes from saline steppes, coastal marshes and rocky cliffs, as in *Limonium* and xerophytes of stony soils and exposed rocks, as in *Acantholimon sensu lato*. A study in the *L. ovalifolium* (*L. lanceolatum*, *L. nydeggeri*, *L. ovalifolium*) and *L. binervosum* (*L. binervosum*, *L. dodartii*, *L. multiflorum*) complexes and in *L. algarvense* using occurrence records on surface lithology encompassing the entire distribution range of the species from each complex, all over the Atlantic coasts in Europe (Ireland, Britain, France, Iberian Peninsula) and in Morocco, revealed that these species typically thrive on sediments and deposits derived from sedimentary rocks like sandstones, conglomerates and mudstones and carbonate rocks as in limestone (Caperta et al., 2017). Whether salt glands evolved as an ecophysiological innovation to regulate Ca concentrations (discussed above) and adapted to secrete a range of ions (see Tables 2 and 3) including those in saline environments is yet to be clarified. In *Armeria*, secretory glands are present both in halophytes and metallophytes found in non-saline sandy soils (e.g., *A. vulgaris*, synonym *A. maritima*; Baumbach & Hellwig, 2007; Ruhland, 1915) and in sub-mountain meadows and mountain pastures (e.g., *A. canescens*; Scassellati et al., 2016). Inland populations of *A. maritima* from sandy and heavy metal soils are salt-tolerant even if less resistant to irrigation with saline water (NaCl) than salt marsh populations (Köhl, 1997). In the related Tamaricaceae, both non-halophytes such as *Myricaria germanica* (Dörken, Parsons, & Marshall, 2017; Table S4) and halophytes such as *Reaumurina* and *Tamarix* species possess salt glands (Abbruzzese et al., 2013; Salama et al., 1999; Weiglin & Winter, 1991; Zhang et al., 2018).

Evolutionary innovations have been correlated with paleopolyploidy (Edger et al., 2015; Soltis & Soltis, 2016; Vanneste, Baele, Maere, & Van de Peer, 2014; Vanneste, Maere, & Van de Peer, 2014), such as the paleopolyploidy event at the base of Portulacineae associated with the evolution of succulence (Edwards & Ogburn, 2012; Nyffeler, Eggli, Ogburn, & Edwards, 2008; Ogburn & Edwards, 2013),

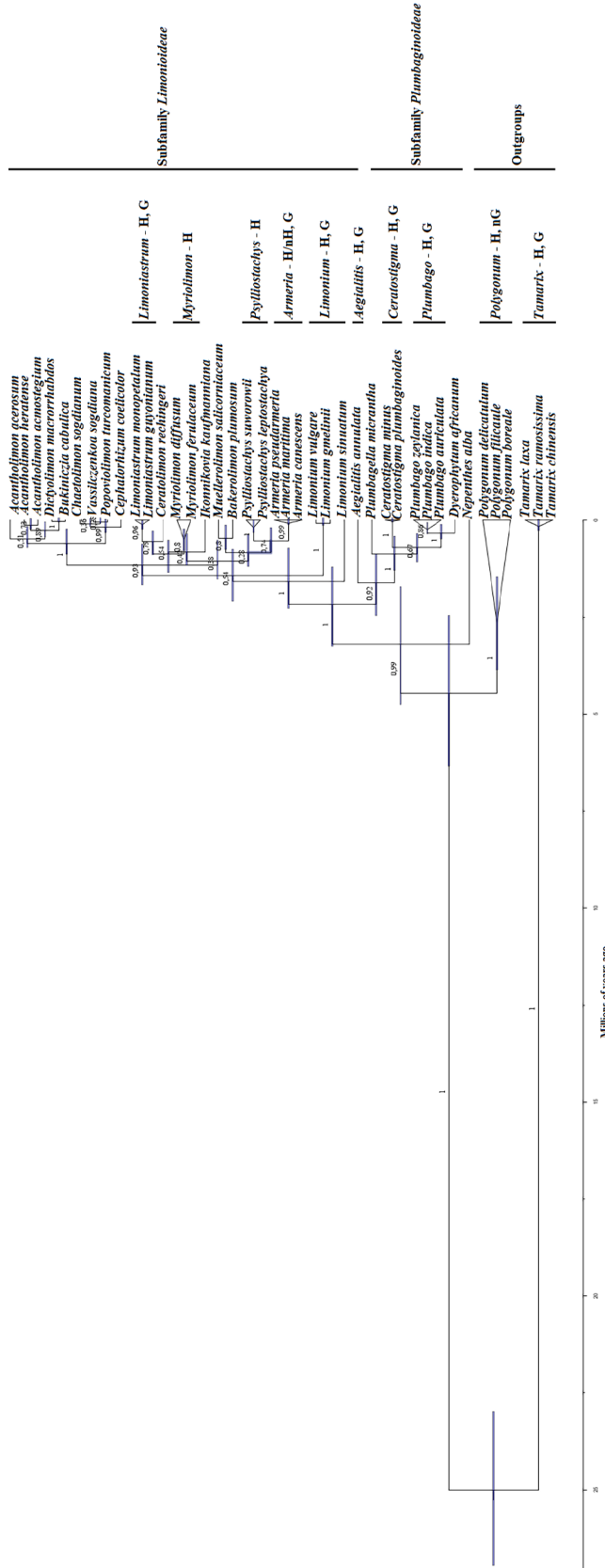


FIGURE 5 Bayesian inference analysis of Plumbaginaceae using chloroplast trnL gene and trnL-trnF intergenic spacer, partial data collected in GenBank (<https://www.ncbi.nlm.nih.gov/>; please see Table S2). Chronogram represents the maximum clade credibility tree estimated in BEAST, with mean divergence dates in million years ago shown for key nodes. Blue bars represent 95% highest posterior density intervals for nodes ages. Values above branches are Posterior Probability. Halophytes (H) and saline glands (G – with glands/nG – without glands), whenever present, information in given for the genus, based on data collected in the bibliographic records (Tables 1–3). Based on the Akaike information criterion calculated in Mega 5.04 (Tamura et al., 2011), general time reversible, GTR + G model of sequence evolution had the best fit to the data. Estimation of phylogenetic relationships and divergence time was conducted using a Bayesian method implemented in BEAST 1.10.4 (Suchard et al., 2018), employing a strict clock model. Along with the GTR + G model of sequence evolution, we used four rate heterogeneity categories and a Calibrated Yule process for speciation model. We calibrated the root node of our tree, using a normal distribution prior with median = 25 Mya and standard deviation (SD) = 0.5 Mya that covers 95% high posterior probability (HPD). A Markov chain Monte Carlo analysis was run for 1,000,000 generations and sampled every 1,000 generations. Tracer v1.7, (Rambaut, Drummond, Xie, Baele, & Suchard, 2018), was used to assess that Effective Sample Size (ESS) were about 200 for optimal convergence and tree likelihood stationarity. A maximum clade credibility (MCC) tree was constructed in TreeAnnotator v.10.4, (Suchard et al., 2018), and the MCC Tree visualized in FigTree v1.4, (<http://tree.bio.ed.ac.uk/>)

or along the branch leading to the Polygonaceae (Schuster et al., 2013) and the branch leading to carnivorous Droseraceae (Rivadavia, Kondo, Kato, & Hasebe, 2003). A phylogenomic sampling based on transcriptomes, show the propensity of polyploidy throughout the evolutionary history of Caryophyllales, and within the non-core Caryophyllales, at least six paleopolyploidy events were inferred (Yang et al., 2018). However, direct connections between genome duplication and biological innovations in the Plumbaginaceae are not yet known.

4 | CONCLUSIONS

The secretory structures that have evolved on the aerial parts of plants can be differentiated between those that secrete organic compounds and those that secrete inorganic ions. All these glands are multicellular structures in dicotyledonous plants, but their ancestry is uncertain. Although the structure of the glands that secrete organic materials is distinct from that of salt glands, it is unclear whether the former gave rise to the latter or if they arose independently. However, salt glands are less common than mucilage glands across the families of flowering plants. For the Plumbaginaceae, the majority of species that have demonstrable tolerance to salt, have functional salt glands (46 of the 54 species included in eHALOPH). Furthermore, the limited data available suggests that even excretion of a small proportion of uptake is critical for salt tolerance. Nonetheless, there are species within the family that have glands structurally similar to functional salt glands in other species, but with untested tolerance to salt as well as some salt-tolerant species where the presence of salt glands has yet to be investigated. The high proportion of halophytic species with salt glands within the family supports the view that ion-secreting glands are essential for salt tolerance in the Plumbaginaceae.

The question we have not been able to answer is whether ion-secreting glands evolved to secrete NaCl and generated salt tolerance or if ion-secreting glands evolve to regulate Ca^{2+} concentrations and this enabled secretion of Na^+ and Cl^- and salt tolerance. Within the Plumbaginaceae there are species that do not grow on saline soils (e.g., *A. caespitosa*, and *A. canescens*, *P. zeylanica*, *P. europaea*, *Limonium sogdianum*, *L. nudum* [gypsum soils], listed in Table 2B), but with glands that look like salt glands (so-called chalk glands). Their presence on non-saline soils gives some weight to the view that glands could have evolved to regulate Ca concentrations in the plant. However, owing to a lack of data we do not know if these species are tolerant of salt or if they have glands capable of secreting Na^+ and Cl^- . Further experimental work could look at the selectivity of glands from species found in different habitats (saline and montane) for different elements (particularly Na and K, but including K and Mg), when grown on media differing in the ratios of these elements; separating the selectivities of roots and glands might be achieved by using both intact plants and detached shoots or leaves.

Most commonly, salt glands in the Plumbaginaceae are formed from 16 cells. Where genera have glands with more than 16 cells, like *Aegialitis* and *Limoniastrum*, they evolved independently as they

form non-sister groups (Figure 3). In the related Frankeniaceae and Tamaricaceae, families where salt tolerance is closely associated with the presence of salt glands, these structures are composed of just eight cells. The absence of a fossil record means that we cannot evaluate the possibility of a common ancestor of the Plumbaginaceae (16-celled glands) and related families of halophytes like non-core Caryophyllales, Frankeniaceae and Tamaricaceae (8-celled glands). The prevalence of salt glands in halophytic members of the Plumbaginaceae, Tamaricaceae and Frankeniaceae and the absence of salt glands in the related Polygonaceae, a family with few halophytes, suggest the evolution of these structures is an aid to salt tolerance. Salt-secreting glands seem to allow the successful colonisation of saline habitats albeit maintained in species thriving in non-saline environments, reflecting the evolutionary independence of this halophytic trait. The presence of such glands also appears to have enabled the colonisation of soils high in heavy metals.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Timothy J. Flowers and Ana D. Caperta coordinated the study. Genorosa Teixeira did the anatomical analyses and Ana Sofia Róis performed the molecular analysis. Pedro Garcia-Caparros tabulated information. Ana D. Caperta and Timothy J. Flowers drafted the manuscript with the input of the co-authors.

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ENDNOTE

¹ Note that different authors use the term “basal cells” to refer to collecting cells, and “accumulating cells” to refer to accessory cells. For simplification we synonymize and use the terms secretory cells, collecting cells (=basal cells) and accessory cells (=accumulating or transfer cells).

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