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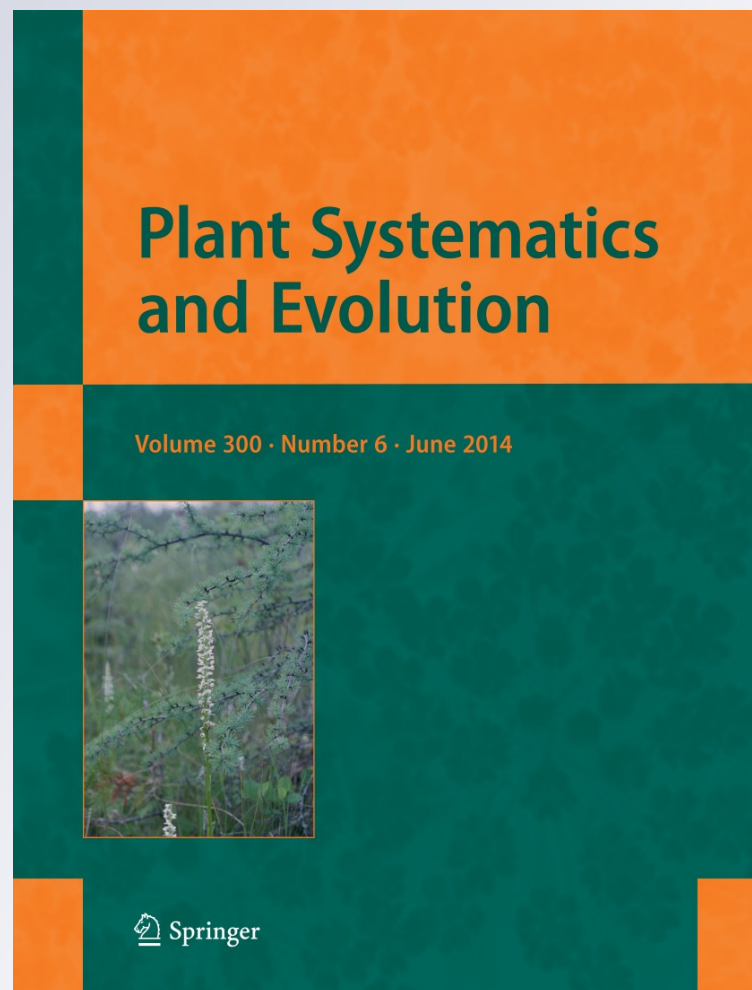
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Anatomical and developmental studies on floral nectaries in *Cardiospermum* species: an approach to the evolutionary trend in Paullinieae

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Abstract Floral nectaries are a widespread trait in the Sapindaceae. However, until now only a few data on nectaries and their evolutionary shifts are available for most taxa. This research focuses on the anatomy and development of floral nectaries in two endemic species, *Cardiospermum heringeri* and *C. integerrimum*. The nectary consists of two horn-like lobes, located at the base of the androgynophore. Anatomically, it is characterized by three components: uniseriate epidermis, sub-epidermal secretory tissue and vascular tissue. The epidermis contains many nectarostomata involved in the exudation process. The secretory parenchyma is composed of small thin-walled cells, relatively lightly stained, and idioblasts containing oxalate druses. Vascular tissue supplying the nectary consists exclusively of phloem. From an early stage of development, the nectary lobes in both species are associated with the base of the posterior petals, but each organ originates independently of one another. These results plus additional morphological observations of nectary lobes in some species of *Cardiospermum*, *Serjania*, *Paullinia* and *Urvillea* were analyzed within the framework of phylogenetic knowledge.

Keywords Floral nectary · Nectary structure · Nectary ontogeny · Paullinieae · *Cardiospermum* · Character evolution

Introduction

Cardiospermum L. (Sapindaceae s.str., tribe *Paullinieae*) comprises 16 species of herbs, vines or suffrutescent herbs (Ferrucci and Urdampilleta 2011) mainly distributed in tropical America, with only *C. corindum* L., *C. grandiflorum* Sw. and *C. halicacabum* L. having an almost cosmopolitan distribution. The genus exhibits two centers of diversity: one in west-central Brazil and the other in north-central Mexico (Ferrucci and Acevedo-Rodríguez 1998). Although *Cardiospermum* has a relatively low number of species, its diversity in morphology and chromosome numbers makes it an interesting genus (Ferrucci 2000a, b; Urdampilleta 2009). The following species occur in South America: *C. anomalum* Cambess., *C. bahianum* Ferrucci and Urdampilleta, *C. cristobaliae* Ferrucci and Urdampilleta, *C. urvilleoides* (Radlk.) Ferrucci, *C. heringeri* Ferrucci, *C. integerrimum* Radlk., *C. oliveirae* Ferrucci, *C. procumbens* Radlk., *C. pterocarpum* Radlk. and the three cosmopolitan species, *C. corindum*, *C. grandiflorum* and *C. halicacabum*.

Within the *Paullinieae* tribe, *Cardiospermum* shares some characters with *Serjania* Mill., *Houssayanthus* Hunz., *Paullinia* L., *Urvillea* Kunth and *Lophostigma* Radlk.: monoecy, unisexual obliquely monosymmetric flowers with a tetramerous corolla, petals with a scale adnate to their adaxial basal surface, a unilateral nectary with two or four protruding lobes, eight stamens of unequal length and fruits that are schizocarps or capsules (Radlkofer 1931–1934; Ferrucci and Anzótegui 1993; Ferrucci 2000a; Weckerle and Rutishauser 2005). Several researchers claimed that *Cardiospermum* is most closely related to *Urvillea*, but the delimitation between both genera is not clear; the former has inflated capsules, no wings or narrow marginal wings, whereas the latter has flattened or

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inflated capsules, with the central locules surrounded by prominent marginal wings (Ferrucci 2000a, b).

Cardiospermum is traditionally divided into three sections: sect. *Cardiospermum* (= *Brachyadenia*), sect. *Ceratadenia* Radlk. and sect. *Carphospermum* Radlk. (Radlkofer 1878). These sections differ in the development of the nectary lobes and the presence or absence of paleaceous hairs on the seed coat. The first phylogenetic scheme of relationships among the South American species of *Cardiospermum* has been performed on the basis of the nuclear markers ITS1 and ITS2 by Urdampilleta et al. (2012). This study showed two well-supported clades comprising species of *Carphospermum* and *Ceratadenia* sections. On the other hand, the analysis of basic chromosome number supports the arrangement of the remaining sections only partially because polyploidy and aneuploidy possibly played a role in karyotype evolution.

Section *Ceratadenia* is represented by *C. heringeri*, *C. integerrimum* and *C. grandiflorum*. *Cardiospermum heringeri* and *C. integerrimum* are both confined to the Atlantic Forest of Brazil, the former to Minas Gerais and Espírito Santo, and the latter to Bahia (Somner and Ferrucci 2009). The three species are closely related; this relationship is supported by molecular analysis, chromosome numbers ($2n = 14$) and morphology (Ferrucci 1993, 2000a; Urdampilleta et al. 2012).

Extrastaminal floral nectaries are widespread in Sapindaceae and represent an apomorphic character that distinguishes the family from others of the order Sapindales (Judd et al. 1999; Ronse Decraene et al. 2000). The nectary morphology ranges from annular to unilateral as a hemi-disc that may be fragmented into four lobes, two anterior and two posterior, or only two posterior ones. The morphology of the floral nectaries may be of systematic importance at the generic or specific level (Radlkofer 1931–1934; Ferrucci 1993, 2000a).

The shape, structure and position of floral nectaries have been used in taxonomic and phylogenetic studies (Solender 1908; Brown 1938; Fahn 1953; Ancibor 1969; Smets 1986; Galetto 1997; Galetto and Bernardello 2004; Bernardello 2007). Particularly, the diversity, structure and development of floral nectaries within Sapindaceae have been scarcely explored. Accordingly, the works of Solís and Ferrucci (2009) and Solís (2011) have been focused on the *Paullinieae* tribe, indicating that the ontogenetic patterns of nectaries show a possible trend wherein an independent origin of nectaries with respect to petal primordia would be a derived trait relative to a simultaneous origin with petals. The aims of this study were to (1) analyze the morphology, anatomy and ontogeny of floral nectaries in the endemic species *C. heringeri* and *C. integerrimum* and (2) assess the evolutionary trend of the number of nectary lobes in the context of the current molecular phylogenetic insights provided in Urdampilleta et al. (2012).

Materials and methods

Open flowers and floral buds of *C. heringeri* and *C. integerrimum* at different developmental stages were fixed in formalin, acetic acid and alcohol (FAA) for anatomical and scanning electron microscopy (SEM) examination. Morphological observations with SEM were performed on flowers of other representatives of *Cardiospermum*, *Paullinia*, *Serjania* and *Urvillea* obtained from specimens fixed in FAA or from herbarium specimens deposited at the Instituto de Botánica del Nordeste herbarium (CTES), Argentina. The species analyzed are listed in Table 1.

For permanent microscope slides, the fixed material was processed by dehydration through an ethanol series with a pre-impregnant rinsing of tertiary butyl alcohol (González

Table 1 Specimens examined and voucher information

Species cited	Voucher	Collection
<i>Cardiospermum anomalum</i> Cambess	Urdampilleta, J. D. & Obando, S. 330 (CTES)	Bahia, Ibotirama, Brazil
<i>C. bahianum</i> Ferrucci and Urdampilleta	Urdampilleta, J. D. et al. 389 (CTES)	Bahia, Rio de Contas, Brazil
<i>C. corindum</i> L. var. <i>corindum</i>	Urdampilleta, J. D. et al. 424 (CTES)	Minas Gerais, Itaobim, Brazil
<i>C. cristobaliae</i> Ferrucci and Urdampilleta	Urdampilleta, J. D. et al. 421 (CTES)	Minas Gerais, Botumirim, Brazil
<i>C. heringeri</i> Ferrucci and Urdampilleta	Urdampilleta, J. D. & Ferrucci, M. S. 437 (CTES)	Santa Teresa, Espírito Santo, Brazil
<i>C. integerrimum</i> Radlk.	Urdampilleta, J. D. & Ferrucci M. S 446 (CTES)	Bahia, Brazil
<i>C. halicacabum</i> L. var. <i>halicacabum</i>	Dematteis, M. et al. 4231 (CTES)	Lavalleja, Uruguay
<i>C. oliveirae</i> Ferrucci	Urdampilleta, J. D. et al. 380 (CTES)	Bahia, Rio de Contas, Brazil
<i>C. pterocarpum</i> Radlk.	Ferrucci, M. S. et al. 2826 (CTES)	Ituzaingó, Corrientes, Argentina
<i>C. urvilleoides</i> (Radlk.) Ferrucci	Urdampilleta, J. D. et al. 425 (CTES)	Minas Gerais, Itaobim, Brazil
<i>Paullinia pachycarpa</i> Benth.	Souza, V. C. et al. 17365 (CTES)	Tapurah, Brazil
<i>Serjania glabrata</i> Kunth	Meza Torres, E. I. et al. 767 (CTES)	Iguazú, Misiones, Argentina
<i>Urvillea peruviana</i> Ferrucci	Ferrucci, M. S. et al. 1112 (CTES)	Cajamarca, Prov. Jaén, Perú
<i>Urvillea triphylla</i> Radlk.	Hatschbach, A. 23335 (CTES)	Paranaguá, Paraná

and Cristóbal 1997) and infiltration in paraffin Histoplast® (Biopack, Buenos Aires, Argentina) according to Johansen (1940). Flowers were sectioned transversely and longitudinally (12 µm thickness) with a rotary microtome; the sections were stained with astra blue-safranin (Luque et al. 1996) and mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). Slides were examined under a Leica DMLB2 (Leica, Wetzlar, Germany) binocular microscope equipped with a digital camera.

For SEM studies, flowers were dehydrated through a series of increasing ethanol solutions. The material was then critical point dried with solvent-substituted liquid carbon dioxide and coated with a thin layer of gold palladium. Micrographs were obtained with a JEOL 5800 LV scanning electron microscope operating at 20 kV.

To reconstruct the evolution of the number of nectary lobes, this character was mapped onto the molecular phylogenetic tree of South American *Cardiospermum* species (Urdampilleta et al. 2012) using the sequence matrix with prior addition of the phenotypic trait of interest. The parsimony analysis was performed using TNT Ver. 1.1 software (Goloboff et al. 2003). The traditional search engine was used with the tree bisection-reconnection (TBR) swapping algorithm, adding 1,000 random addition sequences (RAS) and saving 50 trees per RAS. Bremer support and bootstrap (1,000 replications and traditional search) were used. The trait was then mapped by optimizing the character on the unique most parsimonious tree.

Results

Floral morphology

Flowers of *C. heringeri* are staminate and functionally pistillate (10–12 mm in length). The calyx consists of five

sepals: two external sub-orbicular and three internal, oblong petaloid, with glandular trichomes on the margins. The corolla consists of four petals (8–9 mm in length): two anterior ones form an asymmetrical scale and the two posterior ones form symmetrical scales, which have a deflexed pubescent appendage (Fig. 1a). Two horn-like nectary lobes (ca. 2 mm in length) are located at the base of the androgynophore. The lobes are covered with the scale of the petals throughout flower development, even at blooming time. The nectary persists during the period of fruit maturation, but without the occurrence of secretion (Fig. 1b). The androgynophore is pubescent. Eight stamens are present, with those in the staminate flowers being longer than those in the pistillate flowers. The gynoecium is pubescent and reduced to a pistillode in staminate flowers.

The floral morphology of *C. integerrimum* is similar to that of *C. heringeri*, except for the presence of a glabrous androgynophore and pilose gynoecium with trichomes distributed on the dorsal veins of each carpel in the former species. *Cardiospermum integerrimum* also differs from *C. heringeri* in having glabrous capsule (vs. pubescent), subspherical seeds, 17–20 mm diameter (vs. 4.5–5.5 mm diameter), bony testa (vs. crustaceous) and aril cordate (vs. sub-triangular) (Ferrucci and Urdampilleta 2011).

Structure of nectaries

The general anatomy of the nectary is identical in *C. heringeri* and *C. integerrimum*. Cross sections of nectariferous tissue reveal an epidermis consisting of sub-rectangular and thin-walled cells. Secretory tissue is homogeneous, comprising several layers of small, isodiametric, lightly stained cells with minute intercellular spaces (Fig. 2a–c). Cells with oxalate druses are frequently distributed along the nectariferous tissue. The vasculature consists of phloem

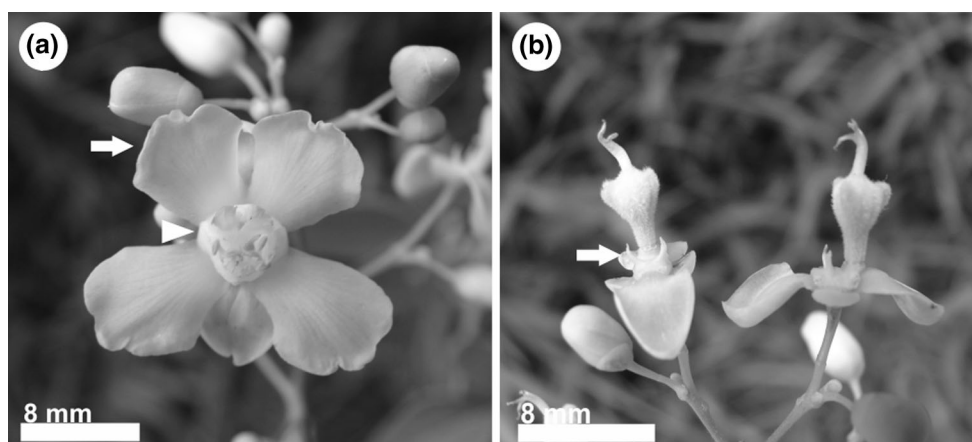


Fig. 1 Photographs of *Cardiospermum heringeri*. **a** Flower at anthesis; the arrow points to a posterior petal and arrowhead to their scale; **b** young fruit with persistent nectary (arrow)

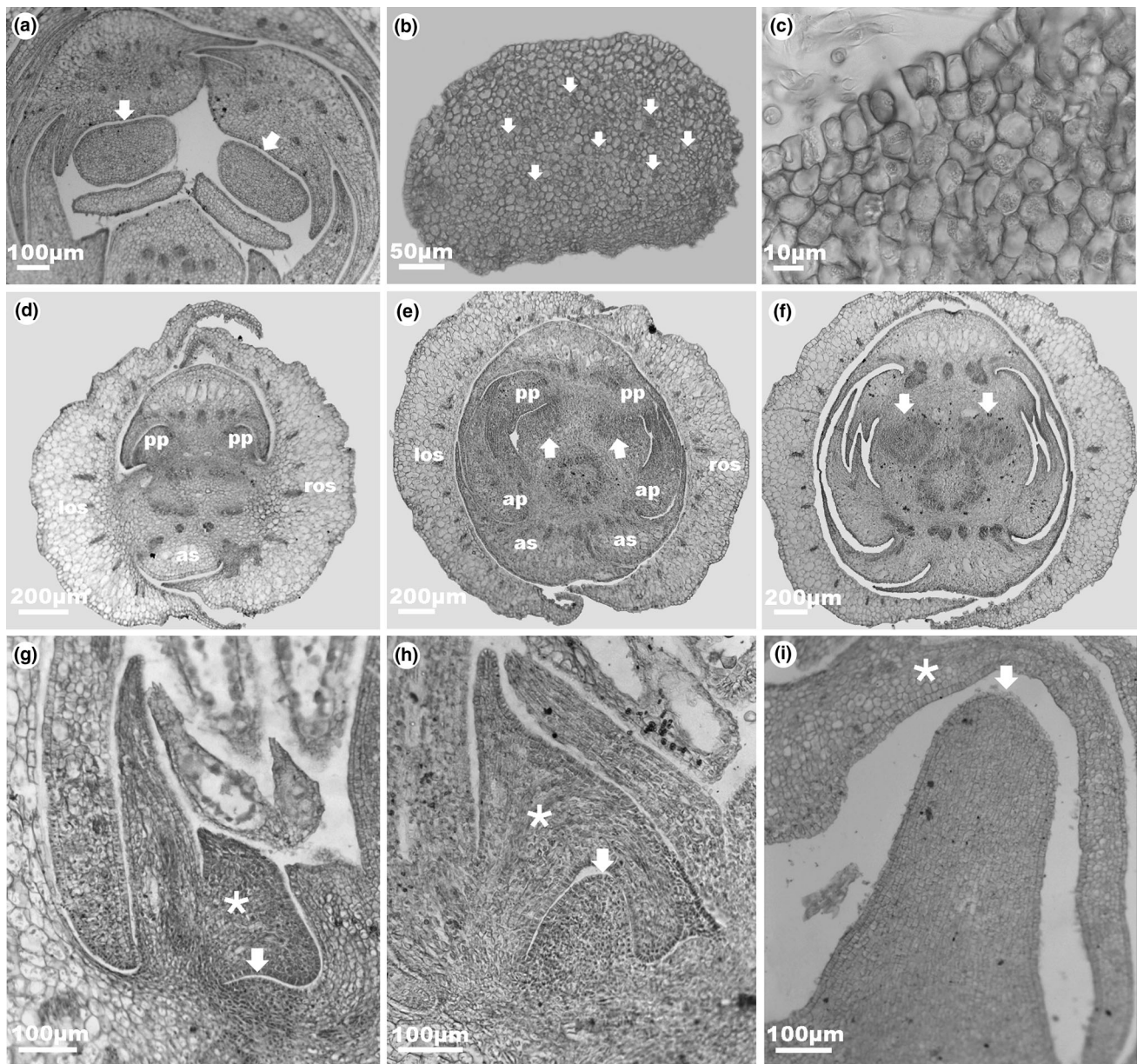


Fig. 2 Light micrographs of *Cardiospermum heringeri*. **a** Cross section of a floral bud; *arrows* point to the two nectary lobes; **b** cross section of a lobe; note the phloem strands (*arrows*); **c** details of nectarostomata and their substomatal chamber. **d–f** Cross sections of floral buds at different stages of development; **d** nectary tissue is still not visible; **e, f** differentiation of nectariferous tissue on the receptacle

(*arrows*). **g–i** Details of longitudinal sections of floral buds at three developmental stages, showing the growth of nectary lobes (*arrows*) and their petal scale (*asterisks*). *as* anterior sepal, *los* left outer sepal, *ap* anterior petal, *pp* posterior petal, *ps* posterior sepal, *ros* right outer sepal

elements derived from the central stele. Between 37 and 50 phloem strands supplying the base of the lobe and between 20 and 27 reaching the apex were observed. These strands are composed of sieve tubes and small companion cells with dense cytoplasm. Nectarostomata are located in depressions, at the same level of the epidermis or slightly raised above the epidermal surface; the substomatal chambers are reduced or absent (Fig. 2c).

Ontogeny

A single description is provided for *C. heringeri* and *C. integerrimum* because the main developmental stages of nectaries are similar in both species. The cross section of a young flower bud of *C. heringeri* shows five developing sepals, two external and three internal ones. One of the internal sepals is posterior and two are anterior. Moreover,

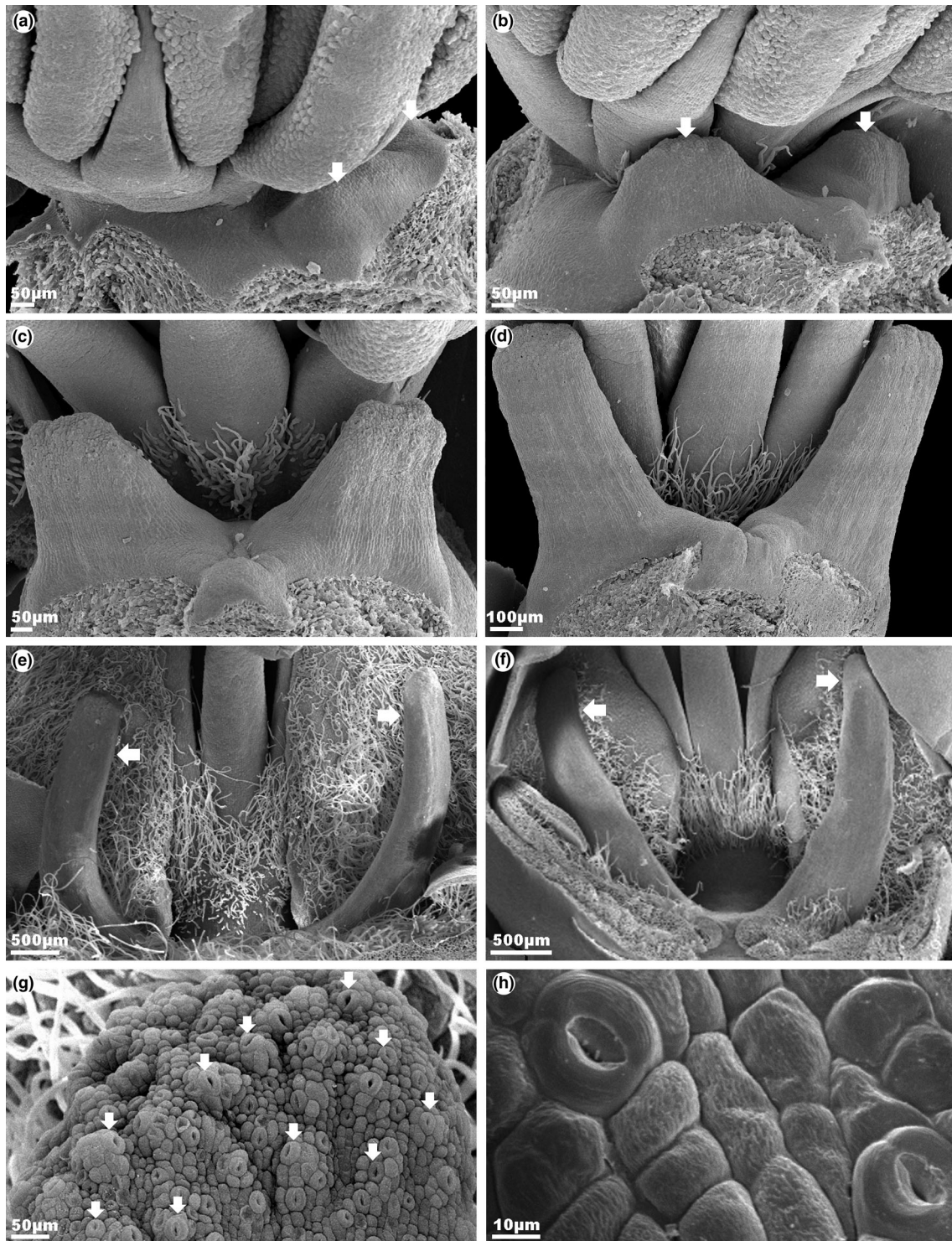


Fig. 3 Scanning electron micrographs of floral nectaries. **a–d** Ontogeny of horn-like lobes (*arrows*) in floral buds of *C. heringeri*, with sepals and petals removed; **e** mature nectary in a staminate flower of

C. heringeri; **f** mature nectary in a staminate flower of *C. integrinum*; **g** details of lobe apex with numerous nectarostomata (*arrows*); **h** details of epidermal cells with cuticle striations and nectarostomata

at this stage, two posterior petals of about 420 μm in length are present between the androgynophore and the posterior sepal, although the nectary tissue is still not visible

(Fig. 2d). Later, the inception of nectary lobes takes place on the receptacle, as shown by two circular areas of cells with a prominent nucleus and dense cytoplasm that were

differentiated in cross section (Fig. 2e). This was also observed with SEM as two dome-shaped protuberances, when the posterior petals had reached at least 1,330 μm in length (Fig. 3a). A gradual increase in diameter and length of nectary lobes occurs during ontogeny along with the increase of parenchymatous tissue that finally connects both lobes and fuses the basal parts of the nectary with the base of the petals (Figs. 2f–i; 3b–e).

In a mature bud, the nectary lobes are fully developed and their surface is mostly glabrous although a small amount of simple hairs are present on the basal portion. Anomocytic nectarostomata are usually open and surrounded by radially oriented epidermal cells with a thinly striated cuticle. Nectarostomata occur mostly on the top of the lobes (Fig. 3g, h), decreasing in quantity toward the base.

Lobe number of floral nectaries in other species of South American *Cardiospermum* and character evolution

SEM observations showed that the nectary lobes in *Carphospermum* section are orbicular to suborbicular, and in *Cardiospermum* section they may be ovoid-obtuse, elliptical or suborbicular. The species of the mentioned sections that have only two nectary lobes are *C. halicacabum*, *C. corindum*, *C. bahianum* and *C. anomalum*. In these species, the posterior lobes present anomocytic nectarostomata. In the species with four lobes, the anterior lobes may be of equal size (*C. pterocarpum*, *C. procumbens* and *C. cristobaliae*) or smaller than the posterior ones (*C. urvilleoides*); in both cases, there are no nectarostomata on the anterior lobe surface.

The number of nectary lobes mapped on the molecular phylogenetic analysis of South American *Cardiospermum* is shown in Fig. 4. In the genera *Paullinia* and *Serjania*, the character is equivocal because some species have four nectary lobes (Fig. 4a, b) whereas other species have only two, although this number is rather infrequent. Two lobes appear to have evolved several times independently of four lobes. In the clade comprising *C. oliveirae* (Fig. 4c), *C. pterocarpum* and *C. procumbens*, a reversal to the ancestral condition occurs in the latter two species. This scenario is the same as in *C. urvilleoides* of the *Cardiospermum* section. The state two nectary lobes is considered to be an apomorphy in *Cardiospermum*. On the other hand, *C. bahianum* and *C. anomalum* (*Carphospermum* section) are closely related, with both having two morphologically similar nectary lobes. Moreover, the three species of the *Ceratadenia* section share the occurrence of a two horn-like nectary lobes (Figs. 2e, f; 4d).

Discussion

Floral nectaries in *Cardiospermum* are of structural type according to the classification system proposed by Zimmermann (1932) and of axial type according to Smets (1988). The most frequent mode of nectar release occurs through stomata in Eudicots (Davis and Gunning 1992; Nepi 2007). This mode of secretion was found in the posterior nectary lobes of the species studied and was also reported for other Sapindaceae, such as *C. grandiflorum*, *Urvillea chacoensis* (Solís and Ferrucci 2009), *Allophylus edulis* (A.St.-Hil.) Hieron. ex Niederl., *Diplokeleba floribunda* N.E.Br., *Melicoccus lepidopetalus* Radlk. and *Koelreuteria elegans* (Seem.) A. C. Sm. subsp. *formosana* (Hayata) F. G. Mey. (unpublished works of Zini et al. 2009; Lezcano et al. 2010; Ávalos et al. 2012). However, the annular nectary of *Koelreuteria paniculata* Laxm. differs in that it lacks nectarostomata on its surface (Ronse Decraene et al. 2000). Vasculature of nectaries in *C. heringeri* and *C. integerrimum* consists of phloem strands that are derived from the receptacle, a result that is consistent with the traits of the above-mentioned species of Sapindaceae. Idioblasts with calcium oxalate crystals in the form of druses are found in the nectariferous parenchyma of both species analyzed. Numerous studies have demonstrated that this feature is rather common in both floral and extrafloral nectaries vascularized by phloem (Durkee 1982; Davis et al. 1988; Davies et al. 2005; Paiva and Machado 2006, 2008).

In general, floral nectaries are useful to explore evolutionary trends in plant groups because they may be reasonably constant characters or may have dramatically changed among groups. In addition, they may be easily lost or acquired within a lineage. The latter possibility contributes to the understanding of the evolution of the group and of its mating systems (Bernardello 2007). Based on our observations and data in the literature, it is evident that nectaries within the tribe *Paullinieae* vary in the number of lobes (two or four) and in shape. In addition, Radlkofer (Radlkofer 1931–1934) described the presence of vestigial anterior lobes, referring to them with the term “obsolete”, in about 12 species of *Serjania* and nine of *Paullinia*. Anterior obsolete lobes may be recognized as structures that are extremely reduced to a ring surrounding the base of the petals, with no vascular strands or nectarostomata. This type of nectary would represent an intermediate state between the four well-developed lobes and the two lobes. In *Cardiospermum* the intermediate state was not observed. However, the anterior lobes that are always lost is a character state shared by this genus with other species of *Paullinieae* that have two nectary lobes. According to the character state, two lobes clearly evolved

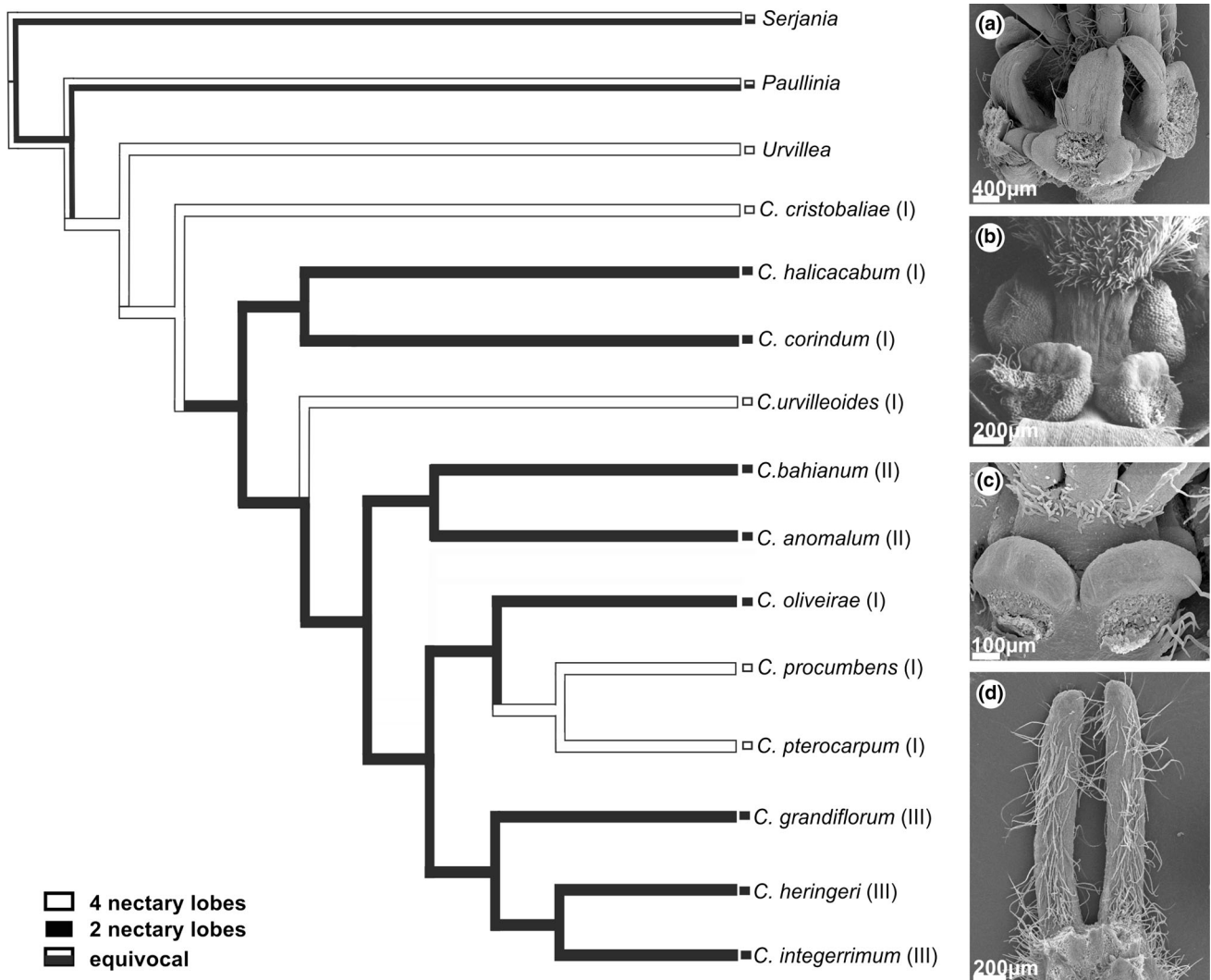


Fig. 4 Inferred evolution of the number of nectary lobes in *Cardiospermum* based on parsimony character mapping onto the phylogeny constructed by Urdampilleta et al. (2012). Species indicated with (I) represents the *Cardiospermum* section, with (II) the representatives

of *Carphospermum* section and with (III) the *Ceratadenia* section. The photographs illustrate character states in **a** *Serjania meridionalis*; **b** *Urvillea triphylla*; **c** *C. anomalum*; **d** *C. oliveirae*; **e** *C. grandiflorum*

independently several times in *Serjania*, *Paullinia* and *Cardiospermum*.

The nectary morphology is of diagnostic value in South American *Cardiospermum*: *Ceratadenia* and *Carphospermum* sections are characterized by the presence of two nectary lobes, and *Cardiospermum* section by two or four lobes, with the posterior lobes equal to or larger than the anterior ones. Therefore, both number and shape of nectary lobes support the current phylogenetic relationships among species of *Ceratadenia* and *Carphospermum* sections. *Ceratadenia* is unique within Sapindaceae in having two nectary lobes with horn-like morphology. The three related species of this section also share the developmental pattern and general nectary anatomy, although the differences lie in the number of nectarostomata and phloem strands, being

noticeably higher in *C. heringeri* and *C. integerrimum* than in *C. grandiflorum*.

Even though in general the number of nectary lobes has been useful to support relationships among most closely related species of the genus *Cardiospermum*, this type of analysis at the intergeneric level would be limited because *Serjania* and *Paullinia* exhibit a similar evolutionary trend to that of *Cardiospermum* (Solís 2011), whereas in *Housayanthus* and *Urvillea* four nectary lobes are always present.

Within the Sapindaceae family, petals and nectary lobes originating simultaneously from a common meristematic group of cells was previously documented in *U. chacoensis* (Solís and Ferrucci 2009). This pattern seems to be the same for *S. meridionalis* Cambess and *P. elegans* Cambess

(Solís 2011). The monophyly of the South American representatives of *Cardiospermum* has been well supported by molecular evidence (Urdampilleta et al. 2012); however, this genus and *Urvillea* can be taxonomically confused due to the slight differences in floral and vegetative characters. An interesting result of the present study is that the independent origin of petals and nectaries is an apomorphic condition that may have evolved within the *Cardiospermum* lineage. Developmental studies also show that changes in the timing of nectary initiation probably resulted in the two pathways of ontogeny mentioned and are likely to play a role in the nectary forms observed among *Paullinieae* tribe. The mechanisms by which the phenotypic traits arose might obscure the assessment of many other morphological evolutionary patterns within *Cardiospermum* or among genera closely related to *Cardiospermum*; therefore, thorough structural and developmental studies will be needed. Our results provide preliminary information for a better understanding of nectary evolution in *Paullinieae*. However, the data remain patchy and further works are necessary to gain comprehensive knowledge of nectary diversity within Sapindaceae.

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