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COMPARATIVE MICROMORPHOLOGY OF PETALS IN MACARONESIAN *LOTUS* (LEGUMINOSAE) REVEALS A LOSS OF PAPILLOSE CONICAL CELLS DURING THE EVOLUTION OF BIRD POLLINATION

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Bird pollination has evolved in four species of Macaronesian *Lotus* from a bee-pollinated ancestor. The transition is associated with the modification of several floral traits, including flower color and size, relative size and orientation of the petals, and nectar composition and quantity. Here, we examine petal surface micromorphology in relation to pollination type, using SEM and LM. In the bee-pollinated *Lotus* (the majority of the genus), papillose conical cells (PCS) are the most abundant epidermal type on dorsal and lateral petals. However, bird-pollinated species completely lack PCS on their dorsal petals and have only a small patch of PCS in a highly localized region of the lateral petal. In the bee-pollinated species (including those most closely related to the bird-pollinated species), PCS develop early in floral development. In contrast, the small amount of residual PCS in bird-pollinated species forms later in development, after the other two major epidermal types have been formed. The almost complete elimination of PCS during the shift of pollination syndrome from bee to birds may be adaptively driven as a both probird and antibee trait.

Keywords: ornithophily, papillose conical cells, petal identity, pollination shift, SEM.

Introduction

Shifts of pollinators require the modification of several floral features associated with attraction, reward of legitimate pollinators, or exclusion of illegitimate pollinators (Cronk and Ojeda 2008). Evolutionary change in pollination syndromes therefore involves the modification of several flower traits (such as flower color, flower size, length of nectar spurs, scent production, and nectar volume and composition). In a few cases, the specific genes involved in transitions have been identified, such as *Anthocyanin 2* (*AN2*) and *flavonoid-3′-hydroxylase* (*F3′H*; Quattrochio et al. 1999; Stuurman et al. 2004; Zufall and Rausher 2004; Galliot et al. 2006; Hoballah et al. 2007) and also major quantitative trait loci (Bradshaw et al. 1998; Bradshaw and Schemske 2003).

Here we study a change in flower morphology that occurred during the evolution of ornithophily (specifically, pollination by opportunistic passerine birds) from melittophilous ancestors (bees and bumblebees) in the papilionoid legumes (Olesen 1985). This change involves a group of four putatively bird-pollinated species within the otherwise bee-pollinated *Lotus* section *Pedrosia* (fig. 1) in the islands of Tenerife and La Palma in the Canary Island archipelago (these bird-pollinated *Pedrosia* species are sometimes referred to as the "rhyncholotus" group, *Lotus* section *Rhyncholotus*). Although there is no unequivocal evidence of the effectiveness of these birds in pollinating *Lotus* species, evidence does exist in the case of *Canarina* and *Isoplexis* (Rodríguez-Rodríguez and Valido 2008, 2011). However, on the basis of the floral morphology and observations of bird visits to the flowers (Sletzer 2005; Ollerton et al. 2009), we consider it highly likely that these species are indeed bird pollinated.

The major differences between the ornithophilous and the melittophilous species are in the following characters: (1) overall size of the flowers, (2) relative size and shape of individual petals, (3) color of flowers, (4) orientation of the flowers and relative orientation of petals within the flower (fig. 1*c*, 1*d*), (5) quantity and composition of nectar, and (6) petal epidermal texture. Floral symmetry has not been altered during the transition (both syndromes have zygomorphic flowers), but the relative size and the role of each petal in pollinator attraction and pollen placement is modified.

The pollination mechanism in the melittophilous species is identical to that described for *Lotus corniculatus* (Proctor et al. 1996). Pollination takes place when a bee lands and pushes down the wings and the keel, forcing out a string of pollen from the stamens located within the keel and resulting in its placement on the underside of the visitor. Bees need a landing platform, and the horizontal position of the flowers allows the lateral petals to play this role (fig. 1*a*).

On the other hand, in the ornithophilous species, the bird seeks the nectar reward located at the base of the calyx, and the pollen is deposited either on the top of the head or on the throat

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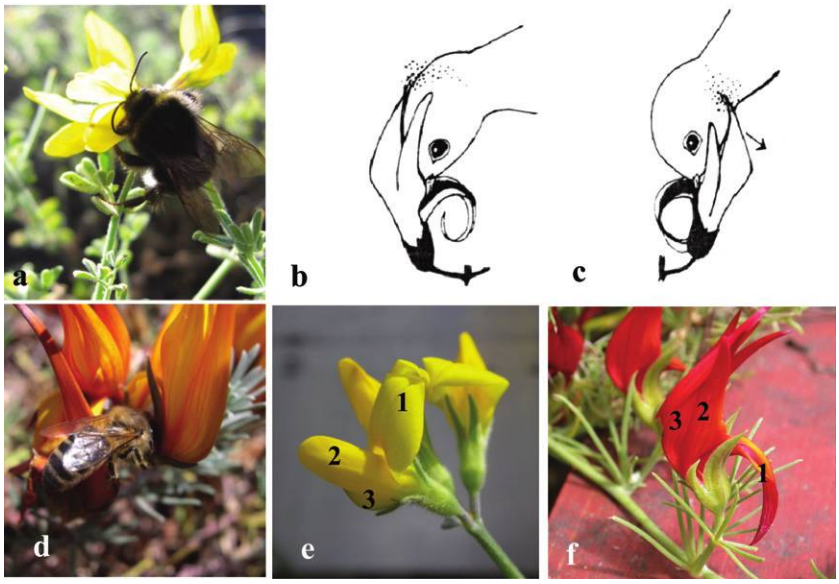

Fig. 1 Bee-pollinated and bird-pollinated species in Macaronesian *Lotus* of section *Pedrosia* s.l. *a*, Bumblebee (*Bombus canariensis canariensis*) foraging in *Lotus hillebrandii* from El Hierro. *b*, *c*, Diagrammatic representation of a passerine bird foraging in a species of rhyncholotus (Olesen 1985). *d*, *Apis mellifera* robbing nectar from *Lotus berthelotii*. *e*, *Lotus sessilifolius*, the closest relative of the bird-pollinated species of rhyncholotus. *f*, *Lotus berthelotii*, one of the four bird-pollinated species. 1, dorsal petal; 2, lateral petal; 3, ventral petal. Photographs by I. Ojeda and Quentin C. B. Cronk.

of the bird when the dorsal petal is pressed down (fig. 1*b*; Olesen 1985). In this case, the bird needs no landing platform (since it usually forages from the ground), and a flower in vertical orientation is better positioned for nectar storage and pollen placement (fig. 1*b*). All four bird-pollinated species have a trailing habit with flowers located close to the ground. To date, only the Canarian chiffchaff, *Phylloscopus canariensis*, and the blue tit, *Parus caeruleus*, have been observed foraging in flowers of only two of these four species (Sletzer 2005; Ollerton et al. 2009), but the similarities of flower morphology among the four species suggest that these birds may visit all four.

The petal modifications in these four bird-pollinated species seem remarkable in the otherwise bee-pollinated tribe Loteae, a group that comprises ~275 species, nearly all with a fairly uniform melittophilous flower morphology. It is the only confirmed case of bird pollination within this tribe. There is another report of a Costa hummingbird visiting *Hosackia rigida*, but the flower morphology of this species is not ornithophilous, and these birds visit this species only in the absence of other bird-pollinated flowers (Grant and Grant 1968). Previous phylogenetic analyses in this group (Allan et al. 2004; Degtjareva et al. 2006) and a recent molecular dating analysis of the group suggest that this pollination syndrome is of recent origin and likely evolved ~1.7 Ma (Ojeda et al. 2011).

In this article, we focus our study on the change of epidermal surface of the standard petal that occurred during the transition to bird pollination. The standard of closely related mellitophilous species is covered with papillose conical epidermal cells (PCS). This cell type is known to be important in bee pollination (Glover and Martin 1998; Martin and Glover 2007; Whitney et al. 2009*a*). It is known to change the optical properties of the petal, increasing the nature and type of reflection (Noda et al. 1994; Gorton and Vogelmann 1996; Whitney et al. 2009*b*) as well as wettability, temperature, and production of volatile compounds (Whitney et al. 2011). Therefore, the loss of papillose epidermal cells (we show that in bird-pollinated species they are flat) may be of functional significance in decreasing visits by bees to bird-pollinated species. Antibee features, as much as probird features, are known to be involved in changes from bee to bird pollination (Castellanos et al. 2004; Cronk and Ojeda 2008).

In *Lotus japonicus*, a *CYCLOIDEA* homologue, *LjCYC2*, specifies dorsal petal identity and is required for the formation of PCS (Feng et al. 2006). If this gene is not expressed, the epidermal cells are flat, whereas all petals develop PCS if it is expressed throughout the flower (Feng et al. 2006). Another gene, *LjCYC3*, acts to specify the identity of lateral petals and is associated with tabular rugose cells (TRS; Feng et al. 2006). In this article, we aim to determine (1) the petal epidermal modifications during the transition from insect to bird pollination and (2) the timing of the differentiation of the major epidermal types during flower development in dorsal, lateral, and ventral petals between closely related species with contrasting flower morphologies.

Material and Methods

Plant Material and Growth Conditions

We examined the petal micromorphology of 56 species. Plants analyzed in this study were obtained from voucher specimens, plants collected in the field, and plants cultivated in three nurseries, either from seeds collected in the wild in the Macaronesian region or from seeds provided by the USDA Agricultural Research Service National Genetic Resources Program (appendix). Plants cultivated at the University of British Columbia were grown in pots of 10–20 cm in diameter at 20°–25°C and were more than 6 wk old when

Table 1

Distribution of Epidermal Types in the Species Analyzed

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
Acmispon:						
Section *Anisolotus*:						
A. americanus	TRS[a]	TRS	TRS	TRS	TFS	TFS
Anthyllis:						
A. hermannie	PCS	PCS	TRS	TRS	TFS	TFS
Coronilla:						
C. valentina	PCS	PCS	PCS	PCS	TRS/TFS	TFS
C. varia	PCS	PCS	PCS	TRS	TFS	TFS
Hosackia:						
H. chihuahuana	PCS	PCS	PCS	TRS	TRS/TFS	TFS
Lotus:						
Section *Bonjeana*:						
L. hirsutus	PCS	PCS	TRS	TRS	TFS	TFS
Section *Chamaelotus*:						
L. glinoides	TRS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Erythrolotus*:						
L. conimbrensis	PCS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Heineckenia*:						
L. arabicus	TRS	PCS	TRS/PCS	TRS	TFS	TFS
L. gebelia	PCS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Krokeria*:						
L. edulis	TRS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Lotea*:						
L. halophilus	TRS	PCS	TRS/PCS	TRS	TFS	TFS
L. weilleri	PCS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Lotus*:						
L. burtii	PCS	PCS	TRS/PCS	TRS	TFS	TFS
L. corniculatus	PCS	PCS	TRS/PCS	TRS	TFS	TFS
L. filicaulis	PCS	PCS	TRS/PCS	TRS	TFS	TFS
L. japonicus Gifu B129	PCS	PCS	TRS/PCS	TRS	TFS	TFS
Section *Pedrosia*:						
L. arborescens	PCS	PCS	PCS	TRS	TFS	TFS
L. arenarius	PCS	PCS	PCS	TRS	TFS	TFS
L. arinagensis	TRS	PCS	TRS	TRS/PCS	TFS	TFS
L. argyrodes	TRS	TRS/PCS	TRS/PCS	TRS	TFS	TFS
L. azoricus	TRS/PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. assakensis	PCS	PCS	TRS/PCS	TRS	TFS	TFS
L. brunneri	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. callis-viridis	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. campylocladus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. creticus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. dumetorum	TRS	PCS	TRS/PCS	TRS	TFS	TFS
L. emeroides	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. eriosolen	TRS	TRS/PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. glaucus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. aff. *spartioides*	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. holosericeus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. hillebrandii	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. jacobaeus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. jolyi	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. kunkelii	TRS[b]	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. lancerottensis	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. latifolius	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. leptophyllus	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. macranthus	TRS	PCS	TRS/PCS	TRS	TFS	TFS
L. maroccanus	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. mascaensis	TRS[b]	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. pseudocreticus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS

Table 1

(*Continued*)

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
L. purpureus	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. salvagensis	PCS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. sessilifolius subsp. *sessilifolius* var. *pentaphyllus*	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. sessilifolius subsp. *sessilifolius*	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. sessilifolius subsp. *villosissimus*	TRS[b]	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. spartioides	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
L. tenellus	TRS	PCS	TRS/PCS	TRS/PCS	TFS	TFS
Section *Rhyncholotus*:						
L. berthelotii	ND[b]	TRS	TRS[b]/PCS	TRS	TRS	TRS
L. eremiticus	ND[b]	TRS	TRS[b]/PCS	TRS	TRS	TRS
L. maculatus	ND[b]	TRS	TRS[b]/PCS	TRS	TRS	TFS
L. pyranthus	ND[b]	TRS	TRS[b]/PCS	TRS	TRS	TRS
Ornithopus:						
O. compressus	PCS	PCS	PCS	TRS/PCS	TFS	TFS
Scorpiurus:						
S. sulcatus	TRS[a]	PCS	TRS	TRS	TFS	TFS

Note. PCS, papillose conical cells; TRS, tabular rugose cells with striations; TFS, tabular flat cells; ND, nondifferentiated. Epidermal types separated by a slash indicate that two epidermal types were observed on the same petal. Underlined values indicate the side of the petal with a higher level of differentiation of epidermal types.

[a] Stomata.

[b] Trichomes.

flowers were collected for analyses. Additionally, we also examined flowers collected from specimens of *Lotus berthelotii* and *Lotus maculatus* that were purchased from commercial nurseries in Vancouver. They were grown under the same environmental conditions as the three bee-pollinated species mentioned above, except for the provision of a vernalization period of 30 d in order to induce flowering.

SEM and LM of Mature Flowers at Anthesis

Fully open mature flowers of each species at anthesis were observed, using a Hitachi S-2600N SEM at 10–12 kV of acceleration voltage. In some cases, fresh flowers in 70% preserved ethanol were examined with LM. Some species were studied from herbarium specimens, in which case they were first rehydrated and preserved in 70% ethanol before observation in LM. The distribution of the cell types was examined for each type of petal (dorsal, lateral, and ventral) on both the adaxial and the abaxial sides. The epidermal types were classified following a previous study of petal epidermal surfaces within the Leguminosae (Ojeda et al. 2009).

We analyzed a total 56 species of seven genera within the tribe Loteae. Within *Lotus*, we analyzed representative species of nine (out of 14) sections currently recognized within this group (Degtjareva et al. 2006). Our analysis included all four bird-pollinated species (the rhyncholotus group of section *Pedrosia*) and 32 (out of 36) of the other currently recognized species within section *Pedrosia* (Sandral et al. 2006), all of which have a bee pollination syndrome (table 1).

Flower Developmental Stages

Flower development in *Lotus japonicus* has previously been divided into seven stages, from stage 0, when floral primordia are initiated, to stage 7, when the primordia of all five petals are initiated but elongation has not started (early stages; Dong et al. 2005). At this stage, the veins and the characteristic epidermal type of each petal type has not yet differentiated (Zhang et al. 2003; Dong et al. 2005; Feng et al. 2006). For this study, we extended the classification of flower development in *L. japonicus* into a further six stages, 8–13 (fig. 2). We considered stages 8–10 as middle developmental stages and stages 11–13 as late developmental stages. Stage 13 is the fully mature open flower. We used morphological landmarks and size relationships of the petals to characterize each stage. Further, analogous stages were established for species in sections *Lotus* section *Pedrosia* and in the bird-pollinated species of the rhyncholotus group, which were analyzed using the same floral developmental characteristics.

Petal Epidermal Surface Differentiation during Flower Development

In order to determine the stage at which the characteristic epidermal micromorphology differentiates on each petal, we also examined with SEM and LM (as described above) the epidermal surface of each petal type during stages 7–13. Epidermal differentiation of each petal was observed in a total of seven species in between three to five biological replicates: *L. japonicus*, *Lotus sessilifolius*, and *Lotus mascaensis* (all bee pollinated) and *Lotus berthelotii*, *Lotus eremiticus*, *Lotus maculatus*, and *Lotus pyranthus* (all bird pollinated). We determined particularly the time of differentiation of papillose conical cells (PCS), TRS, tabular flat cells (TFS), and trichomes in each species. We used analogous stages for each species, following the stages previously established for *L. japonicus*.

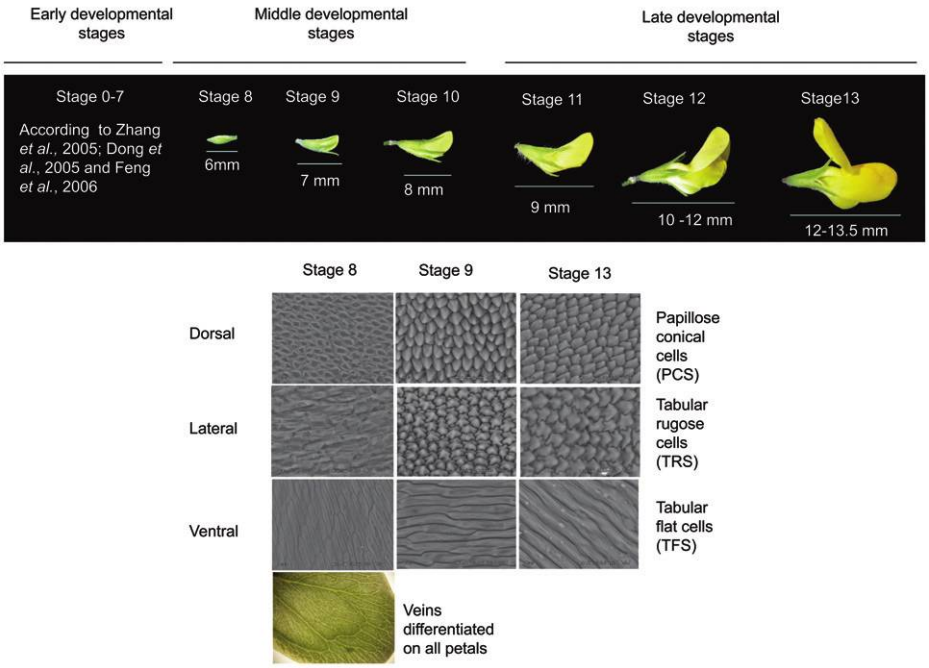

Fig. 2 Differentiation of the major epidermal types during flower development in *Lotus japonicus*. Developmental stages in flowers of *L. japonicus* (stages 8–13). At stage 8, no petal is visible, and all are covered by the sepals. Differentiation of veins and of the characteristic epidermal type of each petal starts at this stage. At stage 9, only the dorsal petal is visible, still folded and completely covering the lateral and ventral petals. The dorsal petal is as long as the sepals. Major and minor veins of each petal are differentiated and evident at this stage. At stage 10, the dorsal petal is still folded and covering the lateral and ventral petals; however, its size has increased, and now the part exposed is as long as the sepals. At stage 11, the dorsal petal is twice the size of the sepals. It is still folded over the lateral and ventral petals, which are now evident. At stage 12, the three types of petals are completely exposed, but their final position in the mature flower has not yet been reached. At stage 13, all petals are fully developed, and the final disposition of each petal in the flower is established.

Reconstruction of Evolutionary Changes

Character evolution was studied using parsimony (DELTRAN) as implemented in MacClade 4.0 (Maddison and Maddison 2000). Traits were coded as binary characters (absence and presence). The distribution of these characters was mapped on a phylogenetic tree of Macaronesian *Lotus* based on four gene regions (Ojeda et al. 2011). We analyzed the following traits: presence or absence of trichomes, presence or absence of PCS in the dorsal petal, and presence or absence of TRS on the dorsal petal (lateralization).

Results

Petal Micromorphology of Bee-Pollinated Flowers within Tribe Loteae

We recorded three major epidermal types in the 56 species analyzed within Loteae. Each epidermal type was mainly restricted to a specific petal type within the flower (fig. 3; table 1), consistent with limited results already reported (Ojeda et al. 2009).

The dorsal petal of most Loteae species is characterized by PCS with striations on both sides (adaxial and abaxial). As a general trend, the adaxial side (the side exposed to pollinators) is more strongly differentiated than the abaxial side (the side not exposed to pollinators; fig. 3; table 2).

The lateral petal is mainly characterized by TRS with striations or by a combination of TRS and PCS. TRS usually covers the majority of the surface of this petal, and it is always located on the basal portion of the petal. PCS, when present, are always restricted to the tip of the petal and cover a greater area on the exposed side (abaxial side) of this petal (fig. 3; table 2). TFS with striations were observed only in the ventral petal. This petal type sometimes has a very small amount of TRS at the base in some species, but they are in the minority compared with TFS. No PCS was recorded on the ventral petal in any of the species analyzed in this study (fig. 3; table 2).

Slight exceptions to this pattern were found in some species (table 2). In the particular case of *Pedrosia*, 14 out of 32 species examined have some TRS on the dorsal petal in addition to PCS (fig. 4*a*). This TRS cell type was found almost exclusively on the abaxial side (i.e., away from pollinators), except in two species where TRS and PCS are mixed on the adaxial side (i.e., the side exposed to pollinators; table 2).

Epidermal Micromorphology in Bird-Pollinated Species

The four bird-pollinated species show a very different pattern of epidermal distribution relative to their bee-pollinated close relatives (fig. 4*a*). These differences are as follows: (1) the dorsal petal completely lacks PCS; (2) in the adaxial side of the dorsal petal PCS is completely replaced by TRS; (3) the abaxial side of the dorsal petal has elongated but poorly differentiated cells (EC), which, apart from the elongation, are more characteristic of early developmental stages before

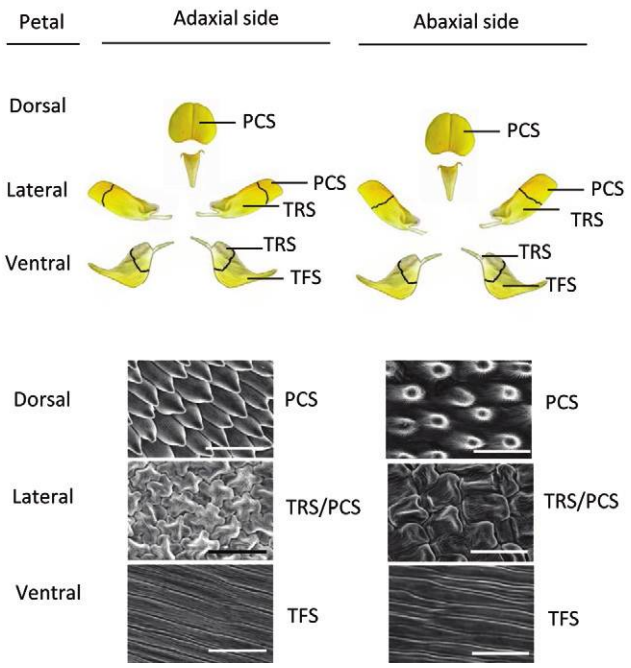

Fig. 3 Major epidermal types recorded in Loteae. *Lotus japonicus* as an example of the distribution of papillose conical cells (PCS), tabular rugose cells (TRS), and tabular flat cells (TFS). The base (claw) of the dorsal petal has been detached from the rest of the petal.

complete differentiation; (4) there is a reduction in the amount of PCS in the lateral petal and a shift in its position to a discrete patch toward the base of the lateral petal; (5) trichomes are present on the abaxial side of the dorsal and lateral petals; and (6) there is an increase in the area covered by TRS in the ventral petal (fig. 4*b*).

Phylogenetic Character Mapping

The complete lack of PCS on the adaxial side of the dorsal petal and the presence of elongated cells on the abaxial side of this petal are unique features of the bird-pollinated species; according to our phylogenetic character mapping analyses, these features are derived in this group, and they evolved only once within Macaronesian *Lotus* (fig. 5*a*).

Presence of Petal Trichomes in Pedrosia *and the Rhyncholotus Group*

Another derived feature of the bird-pollinated species is the presence of trichomes on some of the petals (dorsal and lateral). This feature has apparently evolved at least three times within Macaronesian *Lotus*, and it is also found to a lesser extent (only in the dorsal petal) in three of the species from the related *Lotus sessilifolius* group (table 2; fig. 5*a*).

Trichomes differentiate early, before the three epidermal types are established. Species with trichomes in the dorsal and lateral petals have fully differentiated trichomes at stage 7, while the epidermal types are still undifferentiated.

The four bird-pollinated species have trichomes on both dorsal and lateral petals (fig. 4*b*). Trichomes are evident even with the naked eye and are distributed in lines (between four and six) along the base-tip axis. We found petal trichomes in another four species within *Pedrosia*; however, their density is lower than in the four bird-pollinated species. We also found that the presence of trichomes in these *Pedrosia* species is not consistent among the few individuals we examined. The widely distributed *L. sessilifolius* has petal trichomes in only the population from El Hierro. This population has been considered a separate subspecies (*L. sessilifolius* subsp. *villossisimus*) based (among other morphological features) on the velvet-silvery appearance of the stems and leaves, which are due to a higher presence of trichomes (Sandral et al. 2006). *Lotus assakensis*, although not closely related to the four bird-pollinated species, has been reported to have trichomes on the dorsal petal (Sandral et al. 2006). However, we were unable to find trichomes in the specimens we analyzed. No species in other groups of *Lotus* section *Pedrosia* have ever been reported with petal trichomes.

Petal Micromorphology: Timing of Differentiation during Flower Development

In bee-pollinated species, the characteristic epidermal types (PCS, TRS, and TFS) observed in mature flowers are not present at stage 7 but are established at stage 8 (fig. 2). In *Lotus japonicus*, flower buds at stage 7 (3-mm length) have

Table 2

Classification of the Levels of Lateralization Observed in Bird-Pollinated Members of *Rhyncholotus* and Bee-Pollinated Section *Pedrosia* Species according to the Distribution of the Major Epidermal Types and Trichomes

	Bird pollinated	Bee pollinated	
Modifications	*Rhyncholotus*	*Lotus sessilifolius*[a]	*Lotus japonicus*
Presence of PCS in the dorsal petal	−	+	+
Presence of PCS in the tip of the lateral petal	−	+	+
Increase of TRS in the abaxial side dorsal petal	+	+	−
Trichomes on dorsal petal	+	+	−
Trichomes on lateral petal	+	−	−

Note. Levels of lateralization measured by the presence of tabular rugose cells with striations (TRS). PCS, papillose conical cells; plus sign, present; minus sign, absent.

[a] Another five species also shared these modifications (*Lotus mascaensis*, *Lotus kunkelii*, *Lotus assakensis*, *Lotus loweanus*, and *Lotus chazaliei*).

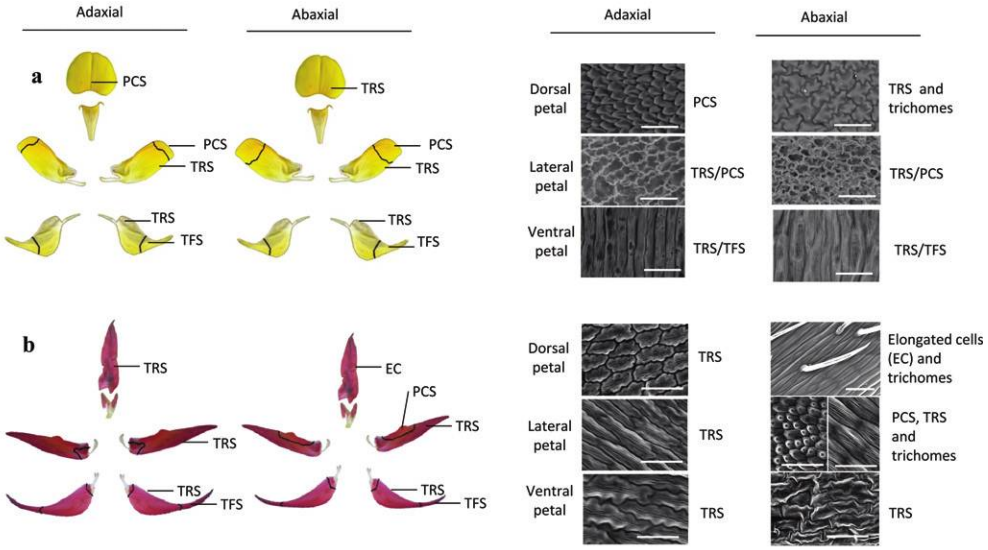

Fig. 4 Distribution of major epidermal types in *Lotus sessilifolius* (and other species within *Pedrosia*; *a*) and *Lotus berthelotii* (and the other three bird-pollinated species; *b*). Dorsal, lateral, and ventral petals are shown. The major epidermal type for each species is shown on the side. PCS, papillose conical cells; TRS, tabular rugose cells; TFS, tabular flat cells; EC, elongated cells relatively undifferentiated. Scale bars = 50 μm.

undifferentiated roughly isodiametric cells, more or less the same in all three types of petals. Differentiation appears to start from the ventral petal and proceed toward the dorsal petal (along a dorsiventral axis; fig. 2).

The bird-pollinated species have a different petal development timing. They have no PCS in the dorsal petal (in sharp contrast to the melittophilous species), but they do have small patches of PCS toward the base of lateral petals on the adaxial side only. PCS develop late, in comparison to the bee-pollinated species, at stage 9 (flower buds between 14 and 16 mm in the four rhyncholotus species). TRS in bird-pollinated species differentiate on the abaxial side of the lateral petal before conical cells start to differentiate on this side, that is, at the same time as in the bee-pollinated species.

Discussion

The Dual Role of Papillose Conical Cells in Lotus *Flowers*

PCS constitute a widespread and common epidermal type in angiosperms and one that has been associated with insect pollination (Kay et al. 1981; Christensen and Hansen 1998). Two distinct roles, optical and mechanical, have been suggested for PCS, which, respectively, (1) alters the properties of light that is reflected from the flower (Glover and Martin 1998, 2002; Comba et al. 2000; Perez-Rodríguez et al. 2005; Martin and Glover 2007) and (2) provides tactile cues (Kevan and Lane 1985) and extra grip in flower handling by bees, thus increasing foraging efficiency (Whitney et al. 2009*a*).

PCS may thus have a different role within the *Lotus* flower, depending on their location. In the dorsal petal, PCS likely has a role in pollinator attraction. The dorsal petal or standard is the most visible petal when an insect approaches the flower. However, when PCS is located at the tip of the lateral petal, it probably aids the insect in handling the petal, therefore increasing foraging efficiency (since the lateral petal in many papilionoids functions as a landing platform for insects). This may explain why PCS, when present on the lateral petals of bee-pollinated flowers, is found only at the tip, where bees are likely to alight on the flower (Proctor et al. 1996).

In contrast, the dorsal petal in the bird-pollinated species does not have the most important role in pollinator attraction (Olesen 1985). Its reflexed position in the flower and small size mean that it does not interfere with beak insertion when a bird is foraging for nectar. Furthermore, this petal completely lacks PCS cells, which have been replaced by TRS. We would expect the adaptive value of PCS in this petal to be reduced in bird-pollinated species, since it is a feature generally associated with bee pollination (Kay et al. 1981). The reflexing of the dorsal petal in bird-pollinated species of *Lotus* is not associated with PCS, contrary to the findings of Baumann et al. (2007) in other plants, in which PCS contribute to petal reflexing.

When foraging on these species, birds do not manipulate the lateral petals to get access to the nectar (Olesen 1985; Ollerton et al. 2009); perhaps for this reason, PCS is also absent from the tips of the petal wings. PCS were, however, observed in a small and highly specific area of the lateral petal. Interestingly, this cell type is restricted to a small but clearly visible and convex area near the mouth of the flower, whose function may be to guide birds to the site of beak insertion (fig. 1*b*, 1*c*, 1*f*). Additionally, PCS has also been associated with volatile production (Whitney et al. 2011), and the different distribution between bee- and bird-pollinated species may also be associated with this. It has been suggested that the multifunctionality of PCS might be explained by the idea of different selective pressures maintaining the presence of PCS in different situations (Whitney et al. 2011). However, in the particular case of Macaronesian *Lotus*, we consider wettability and temperature likely to be less important than pollinator choice, either as pro-bird or antibee, in the maintenance of PCS.

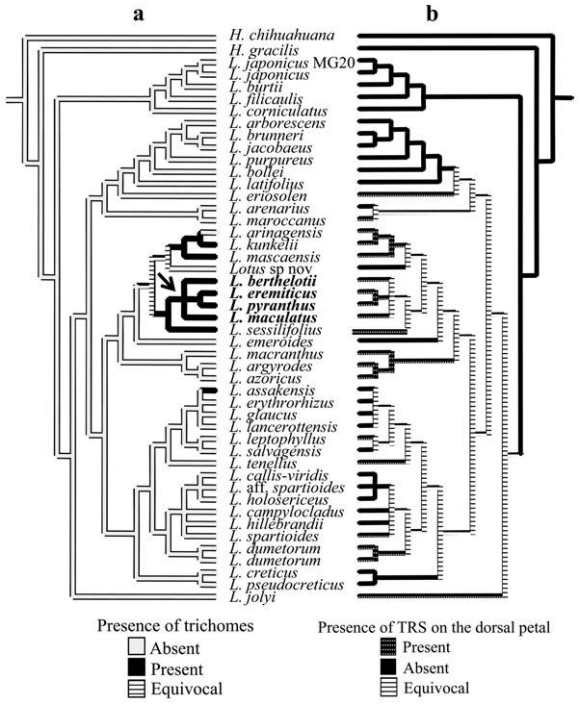

Fig. 5 Character mapping of the evolution of trichomes (*a*) and the presence of TRS in the dorsal petal (lateralization; *b*) in Macaronesian *Lotus* section *Pedrosia* (including the four bird-pollinated species). The arrow indicates the lack of PCS in the dorsal petal in the four bird-pollinated rhyncholotus (in bold), a feature that was lost only once within this group. The traits were mapped using a combined molecular phylogeny based on one nuclear (ITS) and three plastid (*trnH-psbA*, *matK*, and *Cytochrome B6*) gene regions.

The loss of PCS has rarely been documented during pollinator shifts. However, it has been reported during a transition of pollination syndromes from insect to wind in *Thalictrum*. In this genus, conical cells are found in petaloid organs (either sepals or stamens) where these organs have a role in pollinator attraction. Wind-pollinated species have a derived position within this group, and conical cells are absent on the sepals and stamens. Orthologues of a *MIXTA-like2* gene are implicated in this shift (Di Stilio et al. 2009). The presence or absence of PCS in other bird-pollinated flowers has not been fully examined (Christensen and Hansen 1998). In legumes, it is possible that the *LjCYC2* gene exercises control of PCS differentiation by regulating expression of *MIXTA*-like genes, either directly or indirectly.

Loss of Papillose Conical Cells Is Derived in Bird-Pollinated Lotus *and Might Indicate a Shift in Petal Identity*

Our character mapping indicates that the loss of PCS in Macaronesian *Lotus* is derived and occurred only once in the rhyncholotus group (dorsal petal; fig. 5*a*). The lack of this epidermal type in the rhyncholotus group is quite remarkable within the otherwise bee-pollinated *Pedrosia*, where PCS are the dominant epidermal type in the dorsal petal. Papillose cells (including PCS) are commonly found in the exposed side of other legume petals, especially the dorsal petal (Ojeda et al. 2009), and this markedly contrasts with what we found in the dorsal petal of the bird-pollinated rhyncholotus.

In Macaronesian *Lotus*, we found that the decrease in PCS is associated with an increase of TRS in the three types of petals in some groups (fig. 4*a*). This tendency is evident in the closest relatives of the four bird-pollinated species, and the extreme case (lack of PCS on the dorsal petal) was observed in the four bird-pollinated species (fig. 4*b*). Since TRS is more widespread in Macaronesian *Lotus* than bird pollination, the possibility has to be considered that the loss of PCS may be due merely to loss of function mutations fixed by genetic drift. However, the strongly adaptive nature of floral epidermal features (Whitney et al. 2011) makes this unlikely.

In legumes, two petal identity genes of the TCP family of transcription factors have been analyzed in detail in *Lotus japonicus* and *Pisum sativum* (Feng et al. 2006; Wang et al. 2008, 2010). One of these (*LjCYC2*) is required for dorsal petal identity and the differentiation of PCS (Feng et al. 2006). The other petal identity gene (*LjCYC3*) is required for lateral petal development and the differentiation of TRS (Feng et al. 2006; Wang et al. 2010). In contrast with the cell type distribution observed in most Loteae species (similar to *L. japonicus*; table 1; fig. 3), the distribution of epidermal types in the four rhyncholotus suggests that these may have been a shift in the expression of the underlying petal identity genes in the bird-pollinated species. The increase of TRS in the dorsal and ventral petals suggests a "lateralization" of the flower. If the petal identity genes mentioned above are implicated in this shift, we speculate that the lateral identity gene, *LjCYC3*, would have a spatially expanded expression and the dorsal identity gene, *LjCYC2*, should be downregulated. Two *MIXTA*-like genes, *LjMYB1* and *LjMYB2*, are differentially expressed in dorsal and lateral petal domains and are responsible for PCS formation in *L. japonicus* (Weng et al. 2011), but these are under the regulation of *LjCYC* identity genes. Our analyses, therefore, provide a hypothesis at the molecular level that can be tested using closely related species within this group.

Bird-Pollinated Species and the Lotus sessilifolius *Group Share the Presence of Trichomes and the Timing of Their Differentiation*

Our character mapping analysis of the presence of petal trichomes (fig. 5*a*) suggests that this feature has evolved at least two times in *Pedrosia*. Species of the *Lotus sessilifolius* group with trichomes also shared the timing of trichome differentiation with the four bird-pollinated species. We found that trichomes differentiate early during flower development (stage 7) before the major epidermal types—PCS, TRS, and TFS—have been differentiated in the three types of petals. We found, therefore, that most of the species within the *L. sessilifolius* group share not only similar distribution of major epidermal types in mature flowers at anthesis but also developmental similarities during the differentiation of trichomes.

Given the similarities observed in this study, it is likely that the same genetic control is involved in the differentiation of trichomes. Besides the two petal identity genes involved in PCS and TRS differentiation in legumes, orthologues of the *MIXTA* gene are also of particular interest, given that this

transcription factor is involved in trichome and PCS differentiation in *Anthirrhinum majus* (Glover et al. 1998; Perez-Rodríguez et al. 2005) and *L. japonicus* (Weng et al. 2011). The comparative expression of *CYCLOIDEA* and *MIXTA* deserves further investigation, and the Macaronesian *Lotus* represent an ideal system, given both their flower diversity, amenable size, and growing period and the currently available genomic tools, including the nearly complete genome of *L. japonicus* (Sato et al. 2008).

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Appendix

List of Species from Tribe Loteae Used for Petal Micromorphology Analysis in Mature Flowers at Anthesis

Acmispon americanus (Nutt.) Rydb., Cult. UBC PI 215232, *Ojeda 240*/UBC, *Anthyllis hermannie*, UBCBG, no. 035419-0389-2000, *Ojeda 138*/UBC, *Coronilla valentina*, UBCBG, without number, *Ojeda 33*/UBC, *C. varia*, UBC campus, Vancouver, *Ojeda 39*/UBC, *H. chihuahuana*, Cult. UBC PI 18085, *Ojeda 79*/UBC, *Lotus hirsutus* L., UBCBG, no. 032962-0447-1996, *Ojeda 58*, *L. glinoides* Del., Cult. PI 246736, *L. conimbrensis* Brot., Cult. PI 238334, *L. arabicus* L., Cult. UBC PI 214109, *Ojeda 151*/UBC, *L. gebelia* Vent., Cult. PI 464685, *L. edulis* L., Cult. UBC PI 244281, *Ojeda 152*/UBC, *L. halophilus* Boiss. & Spruner, Cult. UBC PI 238336, *Ojeda 153*/UBC, *L. weilleri* Maire, Cult. UBC PI 631729, *Ojeda 154*/UBC, *L. burtii*, Cul. UBC seeds, Miyasaki University, *Ojeda 72, L. corniculatus*, UBC campus, Vancouver, *Ojeda 2*, *L. filicaulis*, Cult. UBC, *Ojeda 71*, *L. japonicus* Gifu B129, Cult. UBC, *Ojeda 70*, *L. arborescens* Lowe ex Cout., Cult. JBCVC # 164/06, *Ojeda 180*/UBC, *L. arenarius* Brot., Cult. UBC PI 631780, *Ojeda 78*/UBC, *L. arinagensis* Brawm., Cult. JBCVC # 49/04, *Ojeda 178*/UBC, *L. argyrodes* R.P Murray, Cult. JBCVC # 5435/UDH/07, *Ojeda 189*/UBC, *L. assakensis* Brand, Voucher, *Jury & Upson 20510*/RNG, *L. azoricus* P. W. Ball, Voucher, ORT # 36336, *L. brunneri* Webb in Hooker, Cult. JBCVC # 514B/07, *Ojeda 181*/UBC, *L. callis-viridis* Bramwell & D.H. Davis, Cult. JBCVC # 145/04, *Ojeda 177*/UBC

L. campylocladus Webb & Berthel., Carretera al Teide, T, *Ojeda 206*/UBC, *L. creticus* L., Cult. JBCVC # 64/07, *Ojeda 188*/UBC, *L. dumetorum* Webb ex R. P. Murray, Mirador Jardina, Anaga, T., *Ojeda 213*/UBC

Ojeda 228/UBC, *L. emeroides* R. P. Murray, Epina, G, *Ojeda 209*/UBC, *L. eriosolen* (Maire) Mader & Podlech, Cult. UBC # PI 631784, Tiznir, Morocco, *L.* aff. *spartioides*, Cañon del Jierro, GC, *Ojeda 174*/UBC, *L. glaucus* Sol., Cult. JBCVC # 223/B/07, *Ojeda 187*/UBC, *L. hillebrandii* Christ, Cumbre nueva a Fuencaliente, P, *Ojeda 232*/UBC, *L. holosericeus* Webb & Berthel., Cult. JBCVC # 334/02, *Ojeda 201*/UBC, *L. jacobaeus* L., Cult. JBCVC 183/06, *Ojeda 179*/UBC

L. jolyi Battand., Voucher, S.L. Jury & Upson 20503/RNG, *L. kunkelii* (Esteve) Bramwell & D. H. Davis, Cult. JCVC # 217/07, *Ojeda 176*/UBC, *L. lancerottensis* Webb & Berth., Voucher, ORT # 37824, *L. latifolius* Brand, Cult. JBCVC 159/06, *Ojeda 183*/UBC, *L. leptophyllus* (Lowe) K. Larsen, Puente de Silva, GC, *Ojeda 171*/UBC, *L. macranthus* Lowe, Voucher, ORT # 36675, *L. maroccanus* Ball, Voucher, *Jury 14471*/RNG, *L. mascaënsis* Burchard, Cult. JBCVC # 442/99, *Ojeda 200*/UBC, *L. pseudocreticus* Maire, Weiller & Wilczek, Voucher, *Davies 53484*/RNG, *L. purpureus* Webb, Cult. JBCVC # 167/06, *Ojeda 184*/UBC, *L. salvagensis*, Voucher, ORT # 35128, *L. sessilifolius* subsp. *sessilifolius* var. *pentaphyllus*, San Juan Guía de Isora, T, *Ojeda 205*/UBC, *L. sessilifolius* subsp. *sessilifolius* D.C., Güimar Polígono industrial, T, *Ojeda 224*/UBC, *L. sessilifolius* D.C. subsp. *villossisimus* (Pitard) Sandral & D.D. Sokoloff, Tecorone, H, *Ojeda 197*/UBC, *L. spartioides* Webb & Berthel., Tamadaba, GC, *Ojeda 217*/UBC, *L. tenellus* (R. Lowe) Sandral, Santos & D.D. Sokoloff, Playa de los Roques, T, *Ojeda 215*/UBC, *L. berthelotii* Masf., Cult. UBC LB-08, Cult. JBCVC 235/07, *Ojeda 238*/UBC, *Ojeda 185*/UBC, *L. eremiticus* A. Santos, Cult. JAO # 100/06, *L. maculatus* Breitf., Cult UBC LM-03

Cult. JBCVC # 187/07, *Ojeda 239*/UBC, *Ojeda 186*/UBC, *L. pyranthus* P. Perez, Cult. JBCVC # 210/99 Cult. JAO # 124-01, *Ojeda 175*/UBC, *Ojeda 226*/UBC, *Ornithopus compressus*, Barranco Madeira, P, *Ojeda 158*/UBC, *Scopiarus fulcatus*, Valle de Masca, T, *Ojeda 160*/UBC

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