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Research article

Bird-pollinated Macaronesian *Lotus* (Leguminosae) evolved within a group of entomophilous ancestors with post-anthesis flower color change

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ABSTRACT

We analyzed the evolution of red/orange flowers in four putatively bird-pollinated species of Macaronesian *Lotus*, with the aim of investigating whether this floral trait evolved from a similar trait found in some entomophilous *Lotus* species, namely the ability to modify flower color to red after anthesis. First, we mapped the ability to modify flower color in this group on a well-resolved and densely sampled phylogenetic tree of the Macaronesian *Lotus*. Secondly, we determined differences in light reflectance and pigment composition between petals of (1) prechange and postchange flowers in bee-pollinated species and (2) between bee and putatively bird-pollinated species. Post-anthesis flower color change evolved three times within Macaronesian *Lotus*, and putatively bird-pollinated species evolved within a clade with this ability to change flower color to red after anthesis. The evolutionary transition to red/orange flowers in the putatively bird-pollinated species involved biochemical changes similar to those of the developmental transition to red postchange flowers. In both cases there are changes in the composition of flavonols and anthocyanidins within the same metabolic pathways, especially in the cyanidin branch of pigment production, but not the activation or inactivation of additional branches of this pathway. Post-anthesis color change in *Lotus*, from yellow to red, is thought to be an adaptation to reduce bee visits to already pollinated flowers. Our results are consistent with the hypothesis that constitutive red coloration for bird-pollination evolved from facultative red flower color change in *Lotus*. As red post-anthesis coloration is widespread in plants, this may possibly represent a widespread exaptive mechanism for the evolution of bird pollination.

Introduction

Oceanic islands offer excellent environments for the study of evolutionary and biogeographic processes (Soja, 1982; Whittaker and Fernández-Palacios, 2007). They are particularly valuable for the study of long distance dispersal, speciation and adaptation, as their ecosystems are in general biologically simpler than their continental counterparts. Like other oceanic islands, the Macaronesian archipelagoes (including Azores, Madeira, Canary, Salvage and Cape Verde islands) offers outstanding examples of endemism and adaptive radiations, e.g. *Echium* (García-Maroto et al., 2009), *Crambe* (Francisco-Ortega et al., 2002), *Aeonium* (Jorgensen and Olesen, 2001; Olesen et al., 2012), *Sonchus* (Kim et al., 1996), *Tolpis* (Mort et al., 2003), *Cistus* (Guzmán and Vargas, 2005) and Campanulaceae (Olesen et al., 2012), among others. Many of these endemics show convergent evolution of insular woodiness and other morphological plant traits.

One such example of parallel evolution is the repetitive convergence of a set of floral traits (i.e. a pollination syndrome), including copious dilute nectar, lack of scent, robust flowers, and red/orange colors, apparently as adaptations to attract opportunistic passerine birds as pollinators (Vogel et al., 1984; Olesen, 1985; Dupont et al., 2004; Valido et al., 2004; Valido and Olesen, 2010). In these islands, floral adaptations to attract opportunistic passerine birds have been proposed in at least 13 endemic species from six plant families (Valido et al., 2004), which represent around 0.4% of the native flora. However, the role of these passerine birds as effective pollinators has been conclusively demonstrated in only two species, *Isoplexis canariensis* (Plantaginaceae) and *Canarina canariensis* (Campanulaceae) (Rodríguez-Rodríguez and Valido, 2008, 2011; Ollerton

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Table 1
Differences in floral traits between bee- and the putatively bird-pollinated species of Macaronesian *Lotus*. Although in some examples floral scent is lost in the transition to bird pollination, in this case both the putatively bird-pollinated species and their closest bee-pollinated relatives appear to be unscented (at least for human detection).

Floral trait	Bee-pollinated	Bird-pollinated
Color	Yellow	Red/orange
Shape	Standard large Keel small	Standard small Keel large
Size	Small	Large
Orientation	Horizontal	Erect
Longevity	Short	Long
Nectar volume	Low	High
Nectar composition	Sucrose	Hexose
Nectar concentration	High	Low
Conical cells in dorsal petal	Present	Absent

et al., 2009; Rodríguez-Rodríguez et al., 2013). Despite the presence of many species with a bird pollination syndrome, there is as yet little understanding of the mechanisms by which all these floral adaptations evolved in such diverse plant lineages in a geographic region that lacks specialist nectarivore birds (e.g. sunbirds and hummingbirds) (Valido et al., 2002, 2004).

Flower color modifications associated with bird pollination have been well characterized in other plant groups (Cronk and Ojeda, 2008; Rausher, 2008). To date most studies at the biochemical level have involved floral transitions to hummingbird pollination while flower color transitions in oceanic island environments, under the selection of opportunistic passerine bird visitors (rather than specialist nectar feeding species) is largely unexplored. Here we address the evolution of red/orange flowers in the "rhyncholotus group" of Macaronesian *Lotus*. The majority of the nearly 40 Macaronesian species within this genus have the usual floral traits (such as yellow petals) found in continental *Lotus*, as in the widespread *L. corniculatus*, and which are strongly associated with bee pollination (Hohmann et al., 1993; Proctor et al., 1996). Floral traits associated with bird pollination are present in four species within this genus (all in the Canary Islands) and the transition from their closest bee-pollinated ancestors (Allan et al., 2004; Degtjareva et al., 2006; Ojeda et al., 2012a,b) involved not only a change from yellow to red/orange flowers, but several additional floral traits, including changes in flower size, shape, orientation, flower longevity, petal micromorphology, as well as nectar composition and concentration that is characteristic of bird pollinated plants (Olesen, 1985; Dupont et al., 2004; Valido et al., 2004; Ollerton et al., 2009; Ojeda et al., 2012a,b) (Fig. 1A and B and Table 1).

Rhyncholotus group species are very rare in the wild so the only direct observations of floral visits by birds, *Phylloscopus canariensis* (Phylloscopidae), are from cultivated plants of *Lotus berthelotii* from Tenerife, Canary Islands (Stelzer, 2005; Ollerton et al., 2009). We have observed honey bees and ants visiting flowers of this group (Ojeda and Santos-Guerra, 2011), but they do not behave as legitimate pollinators. Further studies are needed to determine the effectiveness of passerine birds in pollinating these species. Interestingly, pollination interactions with lizards have also been observed in *Lotus maculatus* (Siveiro and Rodríguez-Rodríguez, 2012) and in *L. berthelotii* (Ollerton et al., 2009) and *Lotus maculatus* (S-RR 2012) (Ollerton et al., 2009). While noting that the circumstantial evidence for bird pollination is very strong we nevertheless refer to this group as "putatively bird-pollinated" because of the lack of direct evidence of pollen transfer by birds. Despite the marked differences in flower morphology between the four putatively bird-pollinated species and their closest bee-pollinated ancestors, the bird pollination syndrome in *Lotus* evolved relatively recently within the last 2 Mya (Ojeda et al., 2012a,b).

One striking characteristic of some bee-pollinated Macaronesian *Lotus* species is their ability to modify flower color after anthesis (Sandral et al., 2006). Flower color change is not unique to the Macaronesian *Lotus* species, as this phenomenon has been observed in other continental *Lotus* species (Weiss, 1995). Observations in other groups suggest that color change is cued by pollination, although unpollinated flowers will also change color, although more slowly (Jones and Cruzan, 1999). We have been able to make extensive observations of color change, both of wild plants in the field in the Canary Islands, as well as of cultivated plants in nurseries and gardens. Flowers after anthesis may modify coloration from yellow/cream (prechange) to brown, pink, orange, purple or red (postchange) depending on the species (Fig. 1D–H). In this study we investigate first whether the four putatively bird-pollinated *Lotus* evolved in lineages with the capacity to modify flower color after anthesis and second, the pigment modifications involved in the transition. In particular we wish to answer the following questions: (1) Is the ability to modify flower color associated with the evolution of red/orange flowers in this group? (2) Are prechange and postchange flowers likely to be perceived differently by pollinators in bee-pollinated species? Similarly, (3) are the putatively bird-pollinated flowers likely to be less discernible by insects than bee-pollinated flowers from the foliage background? (4) What were the modifications in pigment composition and expression of anthocyanin genes during the evolutionary transition in flower color and how does this compare with developmental post-anthesis flower color change? The answers to these questions will then be used to discuss the more general hypothesis that developmental flower color change is a pre-adaptation for the evolutionary transition to bird pollination syndrome.

Materials and methods

Reconstruction of flower color change

Color change was coded as a binary character (absence and presence of flower color change) and the evolution of this trait was mapped on a maximum parsimony phylogenetic tree of the group based on four gene regions (ITS, *Cytochrome B6*, *trnH-psbA* and *matK*) (Ojeda et al., 2012a,b). The phylogenetic analysis analysis gave 451 nearly identical equally parsimonious trees, and for the mapping analysis we selected one of these at random.

Flower color modification after anthesis was analyzed using parsimony (DELTRAN) as implemented in MacClade 4.0 (Maddison and Maddison, 2005). Ancestral state reconstruction was carried out using Mesquite (Maddison and Maddison, 2011). Flower color change was recorded for each species based not only on our own field observations, but also on cultivated plants in botanical gardens (Jardín de Aclimatación de la Orotova and Jardín Botánico Canario "Viera y Clavijo"), and on plants cultivated at the University of British Columbia (UBC) glasshouses. For those species that were neither cultivated nor observed in situ in the field, flower color was obtained from the literature (Monod, 1980; Mader and Podlech, 1989; Brochman et al., 1997; Jardim and Francisco, 2000; Bramwell and Bramwell, 2001; Sandral et al., 2006).

Measurement of petal reflectance spectra

In order to infer the insect visual receptors likely to be stimulated by each flower color, we measured the reflectance of petals of different flower colors. Petal reflectance was measured in situ using fresh collected flowers. Measurements were taken using a portable spectrophotometer (Ocean Optics USB-2000; Duiven,