Pollination of *Habenaria tridactylites* on the Canary Islands

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We investigated the pollination of *Habenaria tridactylites*, an endemic orchid of the Canary Islands. The entirely green, widely open flowers have a long spur containing nectar. We carried out fieldwork, a molecular clock analysis, herbarium surveys, identified pollinators by both morphology and DNA barcoding, and measured the length of floral spurs and insect tongues using a combination of traditional and innovative micro-CT scanning methods to 1) determine the pollinator of this orchid and 2) investigate correlations between local mean spur length and age, altitude and longitude of the island. *Habenaria tridactylites* was found to be pollinated on Tenerife by both small and intermediate sized moth species with variable tongue lengths and mostly belonging to Geometridae and to a lesser extent Crambidae, Erebidae, Noctuidae and Tortricidae. Of the sixteen moth species identified, nine are endemic to the Canary Islands or Macaronesia. The different local populations of *H. tridactylites* on the islands of Gran Canaria, El Hierro, La Gomera, La Palma and Tenerife with different ages and distances from mainland Africa, did not show a significant correlation of mean spur length and altitude, but did show a significant and positive linear correlation with longitude and the geological age of the island. The latter is congruent with the evolutionary arms race theory first proposed by Darwin, suggesting that flowers gradually evolve longer spurs and pollinators longer tongues.

Keywords: endemics, free spur space (FSS), Lepidoptera, orchids, spur, Tenerife

**Introduction**

Orchids display a wide variety of pollination mechanisms (Darwin 1877, Camus 1929, Godfrey and Godfrey 1933, Kullenberg 1961, Nilsson 1981a). In allogamous orchids, the plants rely on animals for the transportation and deposition of pollen. About four-fifth of all orchid species are pollinated by animals, mostly insects (Van der Pijl and Dodson 1966, Grimaldi 1999), although there are various transitions between complete allogamy and some degree of autogamy (Talabj and Brzosko 2008, Claessens and Kleynen 2011, 2012, Jacquemyn et al. 2014). Pollinators may be attracted by various rewards like pollen, nectar, oil or food-hairs (Kull et al. 2009), but in European orchids, nectar is the main reward. Nectar is a key component in the...
relationship between plants and pollinators (Lenaerts et al. 2014), and in orchid flowers it can be secreted on the inflorescences (Subedi et al. 2011), sepals (Subedi et al. 2011, Karremans et al. 2015), lip, lip base or in an elongation of the lip, or the spur (Kocyan et al. 2008). The place where nectar is presented greatly influences the visitor spectrum: an orchid with easily accessible and copious nectar like Neottia ovata (L.) Bluff & Fingerh. attracts a wide variety of pollinators (Claessens and Kleynen 2011, Nilsson 1981b). In contrast, a spur is an effective means of selecting a certain guild of pollinators only. It also acts as a means of enticing the insect to enter the stigmatic cavity, enhancing the chances that it touches the forward projecting viscidium.

Nine European orchid genera (Epipogium J.G.Gmel. ex Borkh., Gymnadenia R.Br., Gennaria Parl., Habenaria Willd., Herminium Guett., Limodorum Böhm., Neottianthe Schltr. and Pseudorchis Ség., Platantlyra L.C.Rich.) from the 31 genera in total produce nectar in a spur, whereas three genera (Anacamptis Rich., Dactylorhiza Neck. ex Nevski and Neotinea Rchb.f.) have only a single species presenting nectar (A. coriophora (L.) R.M.Bateman, D. viridis (L.) R.M.Bateman and N. maculata (Desf.) Stearn). Four of the nectar presenting genera are pollinated by Hymenoptera, four by Lepidoptera and one by Coleoptera (Claessens and Kleynen 2011, 2016). The Hymenopteran pollinated species have a constant spur length, whereas in the Lepidopteran pollinated species spur length can differ considerably. Co-evolution between spur length of orchids and the length of the proboscides of Lepidopteran pollinators is the driving force in the evolution of Lepidopteran pollinated orchid species (Nilsson 1988, Bateman and Sexton 2008, Boberg and Agren 2009, Sletvold and Agren 2010).

The pantropically distributed genus Habenaria (Habenariinae) encompasses about 848 species (Govaerts et al. 2011, Pedron et al. 2012) and has its main centres of diversity in Africa and Meso-America. Most of the species are terrestrial orchids that preferably grow in damp or wet habitats, from low elevations to high montane areas. The main diagnostic features are the often bifid petals, the deeply divided lip and the convex stigma, which can be entire or two-lobed and which often has long, stalked stigmatic lobes (Senghas 1992, Dressler 1993, Pridegon et al. 2001). Most Habenaria species have greenish or pale flowers with nectar secreted in a spur and many species have naked viscidia and long caudicles (Szlachetko and Rutkowski 2000). Scent emission is crepuscular or nocturnal; two characteristics of moth-pollinated flowers. Indeed, most studies refer to moths as their pollinators (Singer 2001, Singer et al. 2007, Pedron et al. 2012, Suetsugu and Tanaka 2014, Ikeuchi et al. 2015, Xiong et al. 2015), followed by butterflies (Moreira et al. 1996) or crane flies (Singer 2001). Pollinaria are deposited on the surface of the eye or on the (base of the) proboscis of the pollinator. Tao et al. (2018) demonstrate the importance of the distance between the viscidia for the place of pollinaria attachment.

Habenaria tridactylites Lindl. is the only representative of Habenaria in Europe and it is endemic to the Canary Islands. The species has two long stigmatic lobes named the stigmaphores (Claessens and Kleynen 2011). The Canary Islands are situated between 27°37’ and 29°25’N and 13°20’ and 18°10’W; its easternmost island Tenerife lies approximately 110 km from the African mainland, whereas El Hierro, the westernmost island, lies at a distance of 474 km from the mainland. The Canaries are famous for their high diversity of plants, with an exceptionally high number of endemics. They are considered a hotspot for plant and animal diversity (Reyes-Betancort et al. 2008) and accommodate 2066–2091 taxa of vascular plants (species and subspecies), of which 536–539 species are endemic, that is 25.6% of the total botanical diversity (Arechavaleta et al. 2010, Aedo et al. 2013). All islands are of volcanic origin; the oldest extant island, Fuerteventura, was formed about 20 million years ago (Mya). The other islands were formed subsequently between 16 and 1.1 Mya (Del-Arco et al. 2006, Steinbauer and Beierkuhnlein 2010, Carracedo and Perez-Torrado 2013) (Fig. 1).

The Canary Islands have never been connected to the African continent. The oldest part of Tenerife is the Roque del Conde volcano, formed between 11.9 and 8.9 Mya. The Teno volcano developed between 6 and 5 Mya, and finally, the Anaga volcano developed between 5 and 4 Mya (Fig. 2). The three shield volcanoes were connected by younger volcanism from the post-erosional felsic Las Cañadas volcano, constructed from about 3.5 Mya (Guillou et al. 2004, Carracedo and Perez-Torrado 2013). The three oldest parts of the island (Roque del Conde, Anaga and Teno) are termed ‘Tenerife old’ in this article. The rest of the island is termed ‘Tenerife young’.

About 730 species of Lepidoptera are known from the Canary Islands (Karsholt and van Nieukerken 2017) of which ca 235 species or subspecies are endemic to the islands, ca 32% (Báez 2010). Noctuidae form the largest family, with 119 species, followed by Geometridae with 75 species.

We hypothesized that the spur length of H. tridactylites would be different on islands with different geological ages and that plants on geologically older islands would have longer spurs. We based our hypothesis on the evolutionary arms race theory first proposed by Darwin (1877). According to this theory, proboscis length of pollinators and floral spur length are reciprocally influenced by positive feedback, leading to a constantly growing elongation of both. This feedback loop is caused by the match between spurs and mean tongue lengths of local pollinator communities that forage on nectar produced in the bottom of the spurs. Too long spurs cause pollinators to lose interest in the orchids as they cannot reach the nectar anymore, too short spurs cause the pollinia to remain in the anther during floral visits. Both changes would result in a loss of reproductive success of the orchids, hence the hypothesized growing spur elongation over time. To collect data to either support or reject this hypothesis, we investigated 1) by which insect species this orchid is pollinated on Tenerife and 2) whether there is a correlation between local mean spur length, age of the island, altitude and longitude on Gran Canaria, El Hierro, La Gomera, La Palma and Tenerife.
Material and methods

Study sites and fieldwork periods

Habenaria tridactylites is the earliest-flowering orchid of the Canary Islands and its flowering period starts in mid-November and can extend to mid-January, depending on the seasonal shifts. We visited Tenerife in 2013, 2014 and 2016 during different periods of the year. The study sites were situated near El Tánque (site 1–3) and Icod de los Vinos (site 4–5) in the north and Los Carrizales (site 6) in the north-western part of the island (Fig. 3).

Del-Arco et al. (2006) distinguish a number of bioclimatic belts on Tenerife. Study site 1–5 were all situated in the north between 600 and 800 m altitude and belonged to the dry to subhumid pluviseasonal thermomediterranean bioclimatic zone under the influence of trade-wind clouds. Vegetation cover was sparse, especially in sites 1, 2 and 3 (El Tánque).
where the orchids grow in a former lava flow resulting from the eruption of the Montaña Negra in 1706 (Solana and Aparicio 1999). Sites 4 and 5 (Icod de los Vinos) were open pine forest with little undergrowth. Accompanying species in all sites were Davallia canariensis (L.) Sm., Pinus canariensis C.Sm. ex DC., Ulex europaeus L. and Erica arborea L. The exotic invasive Centranthus ruber (L.) DC. was present in (close vicinity of) all five sites. Study site 6 was situated in the northwest, at 670 m altitude and belonged to the lower to upper semiarid xeric thermomediterranean bioclimatic zone, without the influence of trade-wind clouds. The vegetation lacked trees but was dominated by Euphorbia atropurpurea Brouss. Other accompanying species were e.g. various Aeonium Webb & Berthel. species, Greenovia aurea (C.Sm. ex Hornem.) Webb & Berthel, Kleinia neriifolia Haw., Sonchus L. sp., Agyranthemum Webb ex Sch.Bip. sp., Monanthes Haw. sp., Geranium molle L. and Opuntia ficus-indica (L.) Mill.; Centranthus ruber was absent from this site.

Habenaria tridactylites is restricted to the northern part of Tenerife, in the zone influenced by the trade winds, providing enough moisture. On the other Canary Islands, the species grows in similar conditions, a sufficient humidity level is a critical condition for the survival of the species. It preferably grows in light shade, although it can also be found in full sun; most localities are exposed to the north. It grows in crevices where some humus has accumulated, on rocky slopes or on moist crags within Laurisilva forests and also within scrublands in less humid environments. It can regularly be found in secondary biotopes like edges of roads or old stone walls surrounding fields, especially in the region of Icod de los Vinos. The plant prefers slightly acid soils. In general, it is the most common orchid of Tenerife and the Canary Islands and can be locally abundant.

In addition to fieldwork, we also surveyed herbarium vouchers of H. tridactylites collected from El Hierro, Gran Canaria, La Gomera, La Palma and Tenerife preserved as dried plants in the herbaria of Naturalis Biodiversity Center in Leiden, the Netherlands and the Natural History Museum of the University of Oslo, Norway (see Supplementary material Appendix 1 Table A1 for more details and Fig. 3 and Table 1 for an overview of all localities from which data were retrieved). In order to see if we could compare data obtained from fresh plants with dried specimens, we measured 17 fresh flowers of H. tridactylites in November 2014 from populations visited by us in El Tanque, dried these and re-measured the dried flowers after two weeks. Data from fresh and dried flowers did not differ significantly (Supplementary material Appendix 1 Table A2), indicating that we could use both fresh and dried plants for our measurements.

Plant measurements

Habenaria tridactylites is 10–40 cm high and has two large ovate, basal leaves. The stem is leafless and carries a lax, cylindrical inflorescence with 2–12 yellowish, odoriferous flowers. The most distinctive feature is the deeply three-lobed lip, giving the species its name (tridactylites means ‘with three fingers’ in Latin). The lateral sepals are spreading; the median sepal forms a hood with the petals. The slender spur is longer than the ovary, pendant and downward curved, containing nectar. The column is short, broad and sloping. The anther cells are wide apart and extend into upward bent prolongations. The spur entrance is placed between the anther cells. The entrance to the spur from the front is hampered by a tongue-shaped outgrowth at the base of the lip. Under the protruding tips of the anther cells lie two fingerlike stigmaphores (Dressler 1981, Szlachetko and Rutkowski 2000, Claessens and Kleynen 2011).

We recorded the length of the spur of the third flower from the base of the inflorescence (Supplementary material Appendix 1 Fig. A1) in both fresh plants in the field and dried plants from herbarium collections. In addition, spur length and accumulated nectar level of all open flowers from ten plants of a short-spurred location (Icod de los Vinos) and
Table 1. Islands and localities where populations of *H. tridactylites* were studied. F = fieldwork; H = herbarium survey; SL = spur length; P = pollination.

| Code+Site | Island         | Longitude  | Latitude   | Field work/herbarium survey | Measurement type |
|-----------|----------------|------------|------------|----------------------------|------------------|
| G1 Teldje | Gran Canaria   | -15.425049 | 27.999277  | H                          | SL               |
| G2 Bandana| Gran Canaria   | -15.459113 | 28.035493  | F                          | SL               |
| G3 Valsequillo | Gran Canaria | -15.501214 | 27.990528  | F                          | SL               |
| G4 Fargas | Gran Canaria   | -15.5617   | 28.02894   | F                          | SL               |
| G5 San Isidro | Gran Canaria | -15.562    | 28.029     | F                          | SL               |
| G6 Los Berrazales | Gran Canaria    | -15.661963 | 28.068977  | F                          | SL               |
| G7 Aegaete | Gran Canaria   | -13.684021 | 28.095430  | F                          | SL               |
| T1 Taborno (Anaga) | Tenerife old | -16.261830 | 28.555203  | H                          | SL               |
| T2 San Diego del Monte (Anaga) | Tenerife old | -16.328255 | 28.501168  | H                          | SL               |
| T3 La Laguna (Anaga) | Tenerife old | -16.346097 | 28.496701  | H                          | SL               |
| T4 Los Carrizales (Teno) | Tenerife old | -16.856676 | 28.315473  | F                          | P                |
| T5 Huente de la Lunz | Tenerife young | -16.37948  | 28.388129  | H                          | SL               |
| T6 Risco de Oro | Tenerife young | -16.536449 | 28.380516  | H                          | SL               |
| T7 Orotava | Tenerife young | -16.545014 | 28.373370  | H                          | SL               |
| T8 Fasnia | Tenerife young | -16.438050 | 28.248553  | F                          | SL               |
| T9 Icod de los Vinos | Tenerife young | -16.700935 | 28.341999  | F                          | P                |
| T10 San Juan del Reparo | Tenerife young | -16.757800 | 28.364091  | F                          | SL               |
| T11 El Tanque | Tenerife young | -16.775856 | 28.354791  | F                          | P                |
| G1 La Gomera | La Gomera | -17.213361 | 28.164907  | H                          | SL               |
| P1 Fuencalcantet | La Palma | -17.850417 | 28.487590  | F                          | SL               |
| P2 San Isidro | La Palma | -17.798141 | 28.635680  | F                          | SL               |
| H1 La Frontera | El Hierro | -18.007956 | 27.748227  | H                          | SL               |
| H2 El Hierro | El Hierro | -17.9775932 | 27.7432053 | H                          | SL               |
| H3 Sabinar | El Hierro   | -18.131423 | 27.754878  | H                          | SL               |

1 Site on island not known.

a long-spurred location (Los Carrizales) on Tenerife were measured. This was measured to obtain an estimate of the proboscis length needed to reach nectar in average flowers. Measurements were all conducted in 2014 and within a few days, in order to avoid seasonal differences.

In order to examine the variations in the volume of the nectar, we measured the spur length and nectar volume of all flowers of a total of 20 plants. Spur length and nectar level in the spur was measured with digital callipers. We also measured the spur length of *C. ruber* on Tenerife, which always accompanied *H. tridactylites* in the northern sites but not in Los Carrizales. In order to investigate if there was a relationship between spur length of *H. tridactylites* and *C. ruber*, we measured spur length of *C. ruber* in two locations on Tenerife, El Tanque (*n* = 31) and Icod de Los vinos (*n* = 24) in 2016 (Supplementary material Appendix 1 Table A5). We also noted the elevation of all sample sites.

**Pollination success**

On one site at Tenerife, we counted the total number of flowers as well as the number of pollinated flowers. Female fitness was calculated by dividing the number of capsules by the total number of flowers.

**Pollinator observations**

Observations were made in the field on Tenerife at three different sites (El Tanque, Icod de los Vinos and Los Carrizales) and took place at various times during the day and between 19:30 and 23:00 h at night. We observed for visitors and pollinators during 11 days (32 h) and 21 evenings (73.5 h) with a total of 105.5 observation hours. All plants observed at night were constantly inspected using a powerful torch and a head lamp. All visitors or pollinators were noted, photographed and caught if possible. No insect was observed during the daytime, whereas observations during night-time proved to be successful. If the visiting insect had pollinaria attached to its body and visited several flowers, it was defined as a pollinator. If the insect inspected the flower but did not carry or remove any pollinaria, it was recorded as a visitor. On 10 out of 21 observation nights we observed visitors or pollinators.

**Insect identifications**

Samples and photographs of specimens that could not be collected were identified morphologically partly by JJB in Santa Cruz de Tenerife, and partly by EJvN in Leiden. Moths brought to Leiden were also dissected to compare the genitalia, and a leg was taken for DNA extraction and DNA barcode assessment. There is no general handbook for all Lepidoptera of the Canary Islands, but a field guide (Báez 1998) allows identification of most larger moths. In addition, genitalia and externals were checked with several sources (Pinker 1965, Klimesch 1987, Hacker and Schmitz 1996, Goater et al. 2005, Fibiger et al. 2010, Witt et al. 2011, Lepiforum e.V. 2017).
**Measurement of proboscis lengths**

Proboscis length of five pollinators was measured in the field using digital calipers (Supplementary material Appendix 1 Fig. A1). All other proboscis lengths were measured by analyzing X-ray photographs of dried museum specimens with the program ImageJ. The X-ray photographs were made by a 3D X-ray with a Sealed transmission 30–160 kV, max 10 W X-ray sources.

Scanning was performed using the following settings: acceleration voltage/power 80 kV/7 W; source current 78.5 μA; exposure time 4–7 s; pictures per sample 1001–1601; camera binning 2; optical magnification 4×, with a pixel size of 1.4–4.7 μm. The total exposure time was approximately 2.5–4 h.

Comparison of measurements of proboscis made in the field (five measurements) to those made with ImageJ showed that there was no significant difference between both, indicating that the program and dried specimens could also be used as a means for measuring proboscis length.

**DNA barcoding of pollinators**

Morphological identifications were also checked with DNA barcodes of 23 specimens. The DNA barcode for animals, the partial cytochrome oxidase subunit 1 (COI) gene was amplified with the primer mixture M13_LepFoLF and M13_LepFoLR (Folmer et al. 1994, Hebert et al. 2004) using the methodology described in Hebert et al. (2003) and in Ratnasingham and Hebert (2007). Details of all barcoded specimens and barcodes, barcode identification numbers (Ratnasingham and Hebert 2013) plus NCBI Genbank accession numbers are provided in Table 2 and in BOLD dataset DS-HABPOL (doi: 10.5883/DS-HABPOL).

**Taxon sampling for molecular clock analysis**

We sampled 279 accessions of 274 orchid species belonging to Habenaria and closely related genera. In addition, we generated new nrITS and matK sequences for Habenaria arenaria Lindl., H. erichmichelii Christenson (= Habenaria rhodocheila Hance), H. macrandra Lindl., H. medusa Kraenzl. and H. tridactylites cultivated at the Hortus Botanicus Leiden, the Netherlands. These sequences were complimented with sequences from published studies in Orchidinae (Inda et al. 2012) and closely related genera. Another calibration point was used to constrain the node of the two endemic species H. tridactylites and G. diphylla to the age of the oldest island, Fuerteventura (20.6 Mya) (Ojeda et al. 2012). We performed two MCMC generations and sampling every 1000 generations and burnin of 10%. We inspected the convergence of independent runs size in Tracer ver. 1.6.

**Statistical analyses**

Data were analysed with one-way ANOVA tests and various posthoc procedures as well as with linear regression analysis using SPSS Statistics 24 to investigate possible correlations between spur length, elevation, longitude and age of the islands, respectively.

**Data deposition**

Data are available from the BoLD Digital Repository: <http://doi.org/10.5883/ds-habpol> (Claessens et al. 2019).
Table 2. Pollinators and visitors of *Habenaria tridactylites* recorded in the field on Tenerife, all belonging to the order Lepidoptera. See also dataset [https://dx.doi.org/10.5883/DS-HABPOL].

| Family          | Species                                                | No. individuals | Pollinator? | Distribution                | Sample sites                 | RMNH registry number                  | BIN                | NCBI GenBank accession no. |
|-----------------|--------------------------------------------------------|-----------------|-------------|-----------------------------|------------------------------|----------------------------------------|--------------------|---------------------------|
| Crambidae       | *Eudonia angustea* (Curtis, 1827)                      | 4+              | yes         | S, SW Europe                | Los Carrizales; El Tanque    | RMNH.5009039; RMNH.5011624; RMNH.5011625; RMNH.5011626 Bacallado s.n. |                    |                           |
| Crambidae       | *Eudonia lineola* (Curtis, 1827)                      | 1              | yes         | S, SW Europe                | El Tanque                   | RMNH.5011610; RMNH.5011611; RMNH.5011627 | BOLD:ADL3576 | MK566741; MK566743; MK566744 |
| Erebidae        | *Eilema albicosta witti* Kobes, 1993                 | 2              | yes         | Endemic Tenerife            | Los Carrizales; Icod de los Vinos | RMNH.5011612; RMNH.5011618 | BOLD:ACD0672 | MK566750; MK566746; MK566746 |
| Erebidae        | *Schrankia costaestrigalis* (Stephens, 1834)         | 3              | yes         | S Europe                    | Los Carrizales               | RMNH.5011610; RMNH.5011611; RMNH.5011627 | BOLD:ADL3576 | MK566741; MK566743; MK566744 |
| Geometridae     | *Ascotis fortunata* (Blachier, 1887)                 | 1              | yes         | Macaronesia                 | Icod de los Vinos            | Bacallado s.n.                         |                    |                           |
| Geometridae     | *Charissa canariensis canariensis* (Rebel, 1911)      | 1              | yes         | Endemic Canary Islands      | Los Carrizales               | RMNH.5011609                          | BOLD:ADL4101 | MK566753                 |
| Geometridae     | *Costaconvexa centrostrigaria* (Wollaston, 1858)     | 1              | yes         | Macaronesia, N America      | Icod de los Vinos            | Bacallado s.n.                         |                    |                           |
| Geometridae     | *Cyclophora maderensis tilineata* (Prout, 1934)      | 34             | yes         | Endemic Canary Islands      | Icod de los Vinos            | Bacallado s.n.                         |                    |                           |
| Geometridae     | *Episaurus kiliani* (Rebel, 1898)                    | 2              | yes         | Endemic Canary Islands      | Icod de los Vinos            | Bacallado s.n.                         |                    |                           |
| Geometridae     | *Gymnoscelis rufifasciata* (Haworth, 1809)           | 1              | no          | Europe                      | El Tanque                   | RMNH.5011616                          | BOLD:ADL3671 | MK566745                 |
| Geometridae     | *Nebula iberica* (Staudinger, 1871)                  | 3              | yes         | SW Europe                   | El Tanque                   | RMNH.5009041; RMNH.5009042; RMNH.5011615 | BOLD:ACF0717 | MK566759; MK566739; MK566755 |
| Noctuidae       | *Cucullia calendae* (Treitschke, 1835)               | 6              | yes         | S Europe                    | El Tanque, Los Carrizales   | RMNH.5009040; RMNH.5011605; RMNH.5011606; RMNH.5011607 | BOLD:ABX5041 | MK566739; MK566755; MK566749; MK566742 |
| Noctuidae       | *Paranataelia whitei* (Rebel, 1906)                 | 1              | no          | Endemic Canary Islands      | Los Carrizales               | RMNH.5011608                          | BOLD:ADL3062 | MK566758; MK566742; MK566742 |
| Tortricidae     | *Clepsis coriacanus* (Rebel, 1894)                  | 1              | no          | Endemic Canary Islands      | Los Carrizales               | RMNH.5011613                          | BOLD:ADL2652 | MK566740                 |
| Tortricidae     | *Acroclita sonchana* Walsingham, 1908               | 3              | yes         | Endemic Canary Islands      | Los Carrizales; El Tanque   | RMNH.5011614; RMNH.5011621; RMNH.5011623 | BOLD:ADL4602 | MK566747; MK566757; MK566748 |
Results

Plant measurements

Mean nectar level was 3.2 ± 2.1 mm (n = 49) at Icod de los Vinos, Los Carrizales and El Tanque on Tenerife. Figure 5D shows that mean spur length was the longest in the easternmost, oldest island (Gran Canaria, n = 86) and the shortest in the two westernmost, youngest islands (La Palma, n = 71 and El Hierro, n = 2). The spur length in Tenerife showed the highest variation for a single island. The longest spurs, comparable to those of Gran Canaria, were found in the old parts of the island, Anaga and Teno, indicated as Tenerife old (n = 120). The shortest spurs were found in Tenerife young (n = 51).

Nectar levels in the spurs of *H. tridactylites* plants differed considerably, both between flowers of a single flower spike as well as between plants. Supplementary material Appendix 1 Figure A1, Table A4 give an indication of the minimal length of the proboscis (in mm) needed to reach the nectar. We found a considerable difference in nectar level, resulting in a variable distance between spur entrance and nectar level, further indicated as free spur space (FSS). This ranged from 15.1% to 153.5%, indicating that in each population there was nectar within reach of even the shortest-tongued pollinators. In the most extreme case, the proboscis length needed for reaching nectar ranged from 4.3 to 10.9 mm. In the short-spurred site (Icod de Los Vinos), pollinators with a proboscis of 4.3 mm long could already reach the nectar. In flowers of the long-spurred site (Los Carrizales), FSS was much higher and nectar could only be reached by pollinators with a proboscis of at least 9.9 mm long. Spur length of *Centranthus ruber* differed between the sites of Tenerife: at El Tanque the length was significantly higher than that of *H. tridactylites* (p = 0.00) whereas in Icod de los Vinos the length of the spurs of both plant species did not differ significantly (p = 0.67) (see also Supplementary material Appendix 1 Table A5).

Fruit set of *H. tridactylites* – in a total of 40 flowers, no signs of autogamy could be detected. In six flowers, one or both pollinaria were still present whereas the flower was already pollinated. Out of 27 plants with 174 open flowers, a
total of 130 flowers (74.1%) were pollinated. On Tenerife the average fruit set was 59.4% (n = 21) (see also Claessens and Kleynen 2011, 2016).

**Pollinators of H. tridactylites on Tenerife**

We never observed a pollinator or a visiting insect during the daytime on any study site. In total, we caught 61 insects of which 53, belonging to 16 different species, were actual pollinators of *H. tridactylites*. All pollinators were Lepidoptera (see Fig. 4, Table 2 for more details). Only on two occasions, we saw a visiting snout moth (Crambidae). Most pollinators belonged to the Geometridae: five different species were noted as pollinators. On one site, we collected 34 specimens of *Cyclorrhapha maderensis trilineata* Prout.

The pollinator spectrum varied between the three sites, due to the different biotopes. Two of the four Geometridae species only seen at Icod de los Vinos feed as caterpillars on *Erica arborea*, only common at this site.

The different pollinator families behaved very differently. Crambidae and Tortricidae sat generally immobile on a flower spike for a long time, from several minutes to more than half an hour. They sometimes moved from flower to flower, may be incited by the light of the torch. The pollinaria were attached to their eyes. In contrast, Geometridae regularly moved from flower to flower and probed the flowers from a few seconds up to several minutes. Pollinaria were generally attached to the proboscis base or sometimes to the eyes. Noctuidae were the most active pollinators, constantly moving from flower to flower. They moved between flower spikes in less than ten seconds. The number of pollinators is underestimated because we were not able to catch all insects during the fieldwork due to the rocky terrain.

Proboscis lengths of pollinators of *H. tridactylites* from Tenerife differed between 4 mm and 15.7 mm and are summarized in Supplementary material Appendix 1 Table A3 and Fig. 5A. Mean spur lengths of *H. tridactylites* from various sites in Gran Canaria, Tenerife, La Palma, La Gomera and El Hierro are summarized in Supplementary material Appendix 1 Table A6 and Fig. 5A. The ANOVA tests indicate that the mean spur length differs significantly for some of the islands (p < 0.000). A non-parametric Kruskal–Wallis test, which is less sensitive for deviations of the requirements of ANOVA, confirms this (p < 0.000). Significant differences were found between El Hierro/La Palma and Tenerife/Gran Canaria (excluding la Gomera) (Supplementary material Appendix 1 Table A7).

**Identification of Lepidoptera**

All identifications are given in Table 2. Unfortunately, since there has not yet been a systematic barcode campaign for Canarian Lepidoptera, for several species no matching barcodes could be found. This resulted in new barcode
identification numbers (BIN’s) for the endemic *Paranataelia whitei* Rebel, *Charissa canariensis canariensis* Rebel, *Acroclita sonchana* Walsingham and *Clepsis coryacamus* Rebel plus new BIN’s for the Tenerife populations of *Evergestis isatidalis* Duponchel and *Gymnoscelis rufifasciata* Haworth. In contrast, the barcodes for *Nebula ibericata* Staudinger, *Cucullia calendulaceae* Treitschke and *Schrankia costaestrigalis* Stephens are not or hardly different from continental European populations.

One specimen that was destructively extracted delivered a barcode with 100% similarity to *Lamoria adaptella* Walker. We consider this an unlikely outcome, as this species is mainly known from Australia and East Asia, with as closest only a single record from Gambia (De Prins and De Prins 2017). Since we cannot confirm the identity, the record is not listed in Table 2, nor on BOLD.

**Molecular clock results**

The most recent common ancestor of *H. tridactylites* and *G. diphylla* was dated to ca 18.5 ± 2.5 Mya (Supplementary material Appendix 1 Fig. A2). This clade was strongly supported by the Bayesian Inference with a posterior probability of 1.0.

**Discussion**

Very little was previously known about the pollination of *Habenaria tridactylites*. There was only one observation of a pollinator recorded by Paulus (1999) on Gran Canaria, identified as possibly *Mamestra brassicae* L. (Noctuidae), with one pollinarium attached to the proboscis base. However, this is an unlikely identification as this species has not been recorded from the Canary Islands (Báez 2010, Vives Moreno

**Figure 5A.** Boxplot of spur length (mm) of *H. tridactylites* per island.

**Figure 5B.** Scatterplot of spur length (mm) of *H. tridactylites* against distance to mainland Africa (measured by longitude).

**Figure 5C.** Scatterplot of spur length (mm) of *H. tridactylites* against mean geological age of the locality of the plant in the field (measured in million years ago).
and close inspection of the photograph shows that this is most likely *Mniotype usurpatrix* Rebel (Noctuidae), a Canarian endemic, common in winter on Gran Canaria (B. Skule unpubl.). During our fieldwork, we discovered that *H. tridactylites* is well suited for pollination by insects. The species is well adapted to night-flying moths: scent emission augments in the afternoon and the pale green coloured flowers are visible when there is little light. The flowers are well accessible to insects: due to the downward arching lip the entrance to the column is wide open. The lateral spreading sepals provide a holdfast for visiting Lepidoptera: they can rest their forelegs on the sepals while searching for nectar (Fig. 4). The long, downward curved spur forces visiting moths to enter the entire proboscis into the spur. The tongue-like ridge on the lip base forces the moths to enter the flower from above. In this position the chances of touching the viscidia are high. If the pollinarium is attached to a pollinator, the caudicle slowly bends forward after being dislodged. This process takes 5–10 min (Claessens and Kleynen 2011) and is a means of preventing self-pollination (Nunes et al 2016). The pollinaria bend downward and inward and are placed in the ideal position for touching the stigmaphores, lying quite close to each other. We regularly saw stigmaphores abutting to each other. Autogamy is not likely, because the anther cells lean backwards and the pollinaria cannot be dislodged without external help.

Pollination efficiency is high, but the coverage of the stigmaphores with pollen varied considerably: some had only a few massulae (pollen packages) adhering, whereas others were covered with many massulae. Pollinators of various Lepidopteran families were observed. Paulus (1999) already found that in Gran Canaria the spur length of *H. tridactylites* flowers differed considerably. Based on the distribution of the spur lengths found, these could be divided in four classes (12–14.4, 14.5–46.9, 17–19.4 and 19.5–21 mm); all spur classes were equally represented, with the exception of the longest spurs. According to Paulus (1999) this is an indication that *H. tridactylites* attracts potential pollinators with different proboscis lengths. Our findings confirm this hypothesis.

Our observations show that *H. tridactylites* is a Lepidopteran pollinated species. In one site *Cyclophora maderensis trilineata* was an abundant and efficient pollinator. This moth is endemic to the Canary Islands and Madeira. It inhabits the laurisilva and adjacent bushes with its hostplant.
tree heath, *Erica arborea* (Fayal-brezial vegetation). It can be found all year round in many annual generations. In one case, we observed six moths, feeding on a group of nine *H. tridactylites* plants. Most pollinators recorded in the North of Tenerife were Lepidoptera commonly associated with the Fayal-brezial (*Myrica faya–Ericion arboreae*), and the majority are also endemic (sub)species.

According to Inda et al. (2012), the divergence between *H. tridactylites* and *G. diphylla* took place in the Miocene, 13–23 Mya. This estimate is in accordance with our own molecular clock analyses inferred from an expanded sampling of *Habenaria* and close relatives (Supplementary material Appendix 1 Fig. A2). Tenerife was formed only 11.9 Mya, so *H. tridactylites* might first have colonised the geological older island Lanzarote and then spread westwards. Alternatively, an ancestral lineage occurring elsewhere might have spread to the Canary Islands after these were formed. Population genetic analyses of *H. tridactylites* and *G. diphylla* are needed to answer this question but that was beyond the scope of this study.

Spur length is an important feature determining which insect can act as a pollinator (Darwin 1877, Nilsson 1988, Maad and Nilsson 2004, Boberg et al. 2014). Yet, spur length is not fixed but depends on various factors. Bateman and Sexton (2008) and Bateman et al. (2012) showed that spur length in European species of the orchid genus *Platanthera* has a latitudinal cline, decreasing northward. Also, spurs of plants in shaded habitats are on average longer than those of plants growing in more open habitats. In our study we found no correlation between altitude (data not shown) and spur length, but a significant linear correlation between island longitude and spur length (Fig. 5B) and between island age and spur length (Fig. 5C). A simple regression of spur length with one explanatory variable (either age or longitude) gives a significant model ($R^2 = 36.8\%$, $p < 0.000$). In both cases, the regression coefficient is significantly positive. A multiple regression with both explanatory variables (either age or longitude) is not applicable to *H. tridactylites* on the Canary Islands as confirmed in this study.

Dispersal of *H. tridactylites* by human activities seems highly unlikely as the plants, seeds and roots do not have any agricultural value and preferably grow in sites that are unsuitable for cultivation. The fact that these orchids are nowadays found in secondary biotopes is caused by increasing human presence on the Canary Islands, reducing the cover of the original biotopes of this orchid species. The seeds are dust-like and can easily travel long distances through the air, enabling colonisation of new biotopes on adjacent islands. Arditti and Ghani (2000) for instance describe that orchids were among the first plants to grow on newly emerging volcanic islands. Therefore, we postulate that *H. tridactylites* spread westwards over the Canary Islands via seed dispersal by wind.
H. tridactylites this mechanism promotes visits of insects with various proboscis length. It also incites insects with a short proboscis to bend over deeply in order to reach the nectar. In doing so they will almost certainly touch the viscid discs and remove the pollinaria. If they already had pollinaria attached, they will press them firmly onto the stigmatic lobes while trying to reach the nectar.

Whereas there seems to be a mismatch in some Habenaria species between pollinator and spur length (Moré et al. 2012), on Tenerife the female success, as expressed in fruit set, is high in both the old and the young parts of the island. Crambidae and Tortricidae had only few pollinaria attached, and seem to be less important pollinators judging from their behaviour. Geometridae were the most abundant pollinators, and five different species acted as pollinators. The largest, fastest-moving pollinators were the Noctuidae, which were exclusively observed in north-western Tenerife (Los Carrizales, geologically belonging to the Teno massif). This is also where the flowers with the longest spurs were found. We do not know if flowers of H. tridactylites in the Anaga mountains are also pollinated by long tongued moths. We did record a long spur length here, but the local pollinator spectrum has not yet been investigated.

When the flowering period of H. tridactylites is ending, another orchid, Gennaria diphylla, starts flowering. Interestingly, we observed that there is an overlap in pollinator spectrum between both orchid species. Several pollinators of H. tridactylites were also observed pollinating the rewarding orchid G. diphylla (Claessens et al. unpubl.). The paucity of co-flowering nectar plants might incite these insects to probe all flowering plants in the area.

During our fieldwork on Tenerife, we noticed that C. ruber was frequently visited by butterflies and moths. This species therefore probably also plays a role in the nectar supply of orchid pollinators. Mean spur length of C. ruber and H. tridactylites on Icod de los Vinos was similar in both species, whereas there was a considerable difference on El Tanque (Supplementary material Appendix 1 Table A5). Given the shortage of flowering plants in the flowering period of H. tridactylites, it might be that the same pollinators visit both species. However, C. ruber is not an indigenous species on the Canary Islands. It is strongly associated with human settlements (Stierstorfer and von Gaisberg 2006). It can have a positive influence on the pollinator spectrum, but it cannot be associated with the evolution of longer spurs over millions of years as assumed for H. tridactylites. It would, therefore, be interesting to investigate whether populations of other plant endemics such as for instance Viola cheiranthifolia Humb. & Bonpl., which is pollinated by bees (Seguí et al. 2017) also have longer spurs on older parts of Tenerife as compared with populations occurring in more recent parts. To the best of our knowledge, correlations in spur length of other plant species and geographical ages of islands have not yet been published. If such data would become available, preferably backed up by real-time divergent evolution experiments, this would provide further support for the theory that either a pollinator shift or evolutionary arms race might be at play between plants and pollinators on the Canary Islands.

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References

Aedo, C. et al. 2013. Species richness and endemcity in the Spanish vascular flora. – Nord. J. Bot. 31: 478–488.

Anderson, B. and Johnson, S. D. 2008. The geographical mosaic of coevolution in a plant–pollinator mutualism. – Evolution 62: 220–225.

Arca, J. and Ghani, K. A. 2006. Numerical and physical properties of orchid seeds and their biological implications. – New Phytol. 145: 365–421.

Arechavala, M. et al. 2010. Lista de especies silvestres de Canarias. Hongos, plantas y animales terrestres. 2009. – Gobierno de Canarias, p. 577.

Báez, M. 1998. Mariposas de Canarias. – Editorial Rueda, S.L.

Báez, M. 2010. Orden Lepidoptera. – In: Arechavala, M. et al. (eds), Lista de especies silvestres de Canarias. Hongos, plantas y animales terrestres. Gobierno de Canarias, pp. 302–318. <http://www.gobiernodecanarias.org/medioambiente/piac/descargas/Biodiversidad/Listas-Especies/Lista_Especies_Silvestres>.

Balbuena, J. A. et al. 2013. PACo: a novel procrustes application to cophylogenetic analysis. – PLoS One 8: e61048.

Bateman, R. et al. 2012. Contrast in levels of morphological versus molecular divergence between closely related Eurasian species of Platanthera (Orchidaceae) suggests recent evolution with a strong allometric component. – New J. Bot. 2: 110–148.

Bateman, R. and Sexton, R. 2008. Is spur length of Platanthera species in the British Isles adaptively optimized or an evolutionary red herring? – Watsonia 27: 1–22.

Batista, J. A. N. et al. 2013. Molecular phylogenetics of the species-rich genus Habenaria (Orchidaceae) in the New World based on nuclear and plastid DNA sequences. – Mol. Phylogen. Evol. 67: 95–109.

Boberg, E. and Ågren, J. 2009. Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid Platanthera bifolia. – Funct. Ecol. 23: 1022–1028.

Boberg, E. et al. 2014. Pollinator shifts and the evolution of spur length in the moth-pollinated orchid Platanthera bifolia. – Ann. Bot. 113: 267–275.

Camus, E. G. 1929. Iconographie des Orchidees d’Europe et du Bassin mediterraneen. vol. 2. – P. Lechevalier, Paris.
Carracedo, J. C. and Perez-Torrado, F. J. 2013. Geological and Geodynamic context of the Teide Volcanic complex. Teide Volcano. – Springer, pp. 23–36.

Claessens, J. and Kleyten, J. 2011. The flower of the European orchid – form and function. – Jean Claessens & Jacques Kleyten.

Claessens, J. and Kleyten, J. 2011. Bestäubung bei Europäischen Orchideen zwischen Allogamie und Autogamie – einige Beispiele. – Ber. Arbeitskreis. Heim. Orchid. Beiheft 8: 14–31.

Claessens, J. and Kleyten, J. 2016. Orchidées d’Europe, fleur et pollinisation. – Biotope Editions.

Claessens, J. et al. 2019. Data from: pollination of Habenaria tridactylites on the Canary Islands. – BoLD Digital Repository, <http://doi.org/10.5883/ds-habpol>.

Darwin, C. 1877. The various contrivances by which British and foreign orchids are fertilised by insects. – John Murray.

De Prins, J. and De Prins, W. 2017. Afromoths, an online database of Afrotropical moth species (Lepidoptera). – Belgian Biodiversity Platform. <http://www.afromoths.net/>, accessed 20 February 2018.

Del-Arco, M. et al. 2006. Bioclimatology and climatophilous vegetation of Tenerife (Canary Islands). – Ann. Bot. Fenn. 43: 167–192.

Déniz-Páez, J. et al. 2012. Quantitative size classification of scoria cones: the case of Tenerife (Canary Islands, Spain). – Phys. Geogr. 33: 514–535.

Dole, J. and Doyle, J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. – Phytochem. Bull. 19: 11–15.

Dressler, R. L. 1981. The orchids. – Harvard Univ. Press.

Folmer, O. et al. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. – Mol. Mar. Biol. Biotechnol. 3: 294–299.

Garth, J. S. and Tilden, J. W. 1986. California butterflies. – Univ. of California Press.

Goater, B. et al. 2005. Pyraloidea 1 (Crambidae: Acentropinae, Evergestinae, Heliothelinae, Schenoniinae, Scopariinae). Microlepidoptera of Europe. – Apollo Books.

Godfrey, M. and Godfrey, H. 1933. Monograph and iconograph of native British Orchidaceae. – Univ. Press.

Govaerts, R. et al. 2011. World checklist of Orchidaceae. – The Board of Trustees of the Royal Botanic Gardens, Kew. <http://apps.kew.org/wcsp/>.

Grimaldi, D. 1999. The co-radiations of pollinating insects and angiosperms in the Cretaceous. – Ann. Miss. Bot. Gard. 86: 373–406.

Guillou, H. et al. 2004. Implications for the early shield-stage evolution of Tenerife from K/Ar ages and magnetic stratigraphy. – Earth Planet. Sci. Lett. 222: 599–614.

Hacker, H. and Schmitz, W. 1996. Fauna und Biogeographie der Noctuidae des makaronischen Archipels. – Esperiana 4: 167–221.

Hebert, P. D. et al. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. – Proc. Natl Acad. Sci. USA 101: 14812–14817.

Hebert, P. D. et al. 2003. Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. – Proc. R. Soc. B 270: 596–599.

Hodges, S. and Whittall, J. 2008. One-sided evolution or two? A reply to Ennos. – Heredity 100: 541–542.

Ikeuchi, Y. et al. 2015. Diurnal skipper Pelopidas mathias (Lepidoptera: Hesperiidae) Pollinates Habenaria radiata (Orchidaceae). – Entomol. News 125: 7–12.

Inda, L. A. et al. 2012. Phylogenetics of tribe Orchideae (Orchidaceae: Anthocerotidae) based on combined DNA matrices: inferences regarding timing of diversification and evolution of pollination syndromes. – Ann. Bot. 110: 71–90.

Jacquemyn, H. et al. 2014. Biological flora of the British Isles: Epipactis palustris. – J. Ecol. 102: 1341–1355.

Karremans, A. P. et al. 2015. Pollination of Specklinia by nectar-feeding Drosophila: the first reported case of a deceptive syndrome employing aggregation pheromones in Orchidaceae. – Ann. Bot. 116: 437–455.

Karsholt, O. and van Nieukerken, E. 2017. Fauna Europaea: Lepidoptera. – Fauna Europaea, ver. 2017.06. <https://fauna-eu.org/>.

Kearse, M. et al. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. – Bioinformatics 28: 1647–1649.

Kisel, Y. et al. 2012. Testing the link between population genetic differentiation and clad diversification in Costa Rican orchids. – Evolution 66: 3035–3052.

Klimesch, J. 1987. Beitrag zur Kenntnis der Microlepidopterenfauna des Kanarischen Archipels. 9. Beitrag: Tortricidae, Cochylidae. – Viera 17: 297–322.

Kocyan, A. et al. 2008. Molecular phylogeny of Aerides (Orchidaceae) based on one nuclear and two plastid markers: a step forward in understanding the evolution of the Aeridinae. – Mol. Phylog. Evol. 48: 422–443.

Kull, T. et al. 2009. Orchid biology: reviews and perspectives X. – Springer.

Kullenberg, B. 1961. Studies in Ophry pollination. – Almqvist and Wiksell's Boktryckeri AB.

Lenaerts, M. et al. 2014. Rosenbergiella australoborealis sp. nov., Rosenbergiella collisarenosi sp. nov. and Rosenbergiella epipactidis sp. nov., three novel bacterial species isolated from floral nectar. – Syst. Appl. Microbiol. 37: 402–411.

Lepiforum e.V. 2017. Bestimmungshilfe für die in Europa nachgewiesenen Schmetterlingsarten. – <http://www.lepiforum.de/lepiwiki.pl?Bestimmungshilfe>, accessed 28 September 2017.

Maad, J. and Nilsson, L. A. 2004. On the mechanism of floral shifts in speciation: gained pollination efficiency from tongue-to-eye-attachment of pollinia in Platanthera (Orchidaceae). – Biol. J. Linn. Soc. 83: 481–495.

Miller, M. A. et al. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 gateway computing environments workshop (GCE). – IEEE, pp. 1–8.

More, M. et al. 2012. Armament imbalances: match and mismatch in plant–pollinator traits of highly specialized long-spurred orchids. – PLoS One 7: e41878.

Moreira, G. R. et al. 1996. Pollination of Habenaria pleiophylla (Orchidaceae) by Drosophila: the first reported case of a deceptive syndrome employing aggregation pheromones in Orchidaceae. – PLoS One 7: e41878.
Nilsson, L. A. 1981a. Pollination ecology and evolutionary processes in six species of orchids. – Almqvist and Wiksell Int.

Nilsson, L. A. 1981b. The pollination ecology of *Listera ovata* (Orchidaceae). – Nord. J. Bot. 1: 461–480.

Nilsson, L. A. 1988. The evolution of flowers with deep corolla tubes. – Nature 334: 147–149.

Nilsson, L. A. 1998. Deep flowers for long tongues. – Trends Ecol. Evol. 13: 259–260.

Nunes, C. E. et al. 2016. The dilemma of being a fragrant flower: the major floral volatile attracts pollinators and florivores in the euglossine-pollinated orchid *Dichaea pendula*. – Oecologia 182: 933–946.

Ojeda, I. et al. 2012. The origin of bird pollination in *Macaronesian lotus* (Loteae, Leguminosae). – Mol. Phylogeny. Evol. 62: 306–318.

Paulus, H. F. 1999. Bestäubungsbiologische Untersuchungen an *Oporys bombyliflora*, *Orchis canariensis* und *Habenaria tridactylites* (Orchidaceae) in Gran Canaria (Spanien). – Ber. Arbeitskrs. Heim. Orchid. 16: 4–22.

Pauw, A. et al. 2009. Flies and flowers in Darwin’s race. – Evolution 63: 268–279.

Pedron, M. et al. 2012. Pollination biology of four sympatric species of *Habenaria* (Orchidaceae: Orchidinae) from southern Brazil. – Bot. J. Linn. Soc. 170: 141–156.

Pérez-Escobar, O. A. et al. 2016. Rumbling orchids: how to assess divergent evolution between chloroplast endosymbionts and the nuclear host. – Syst. Biol. 65: 51–65.

Pérez-Escobar, O. A. et al. 2017. Recent origin and rapid speciation of Neotropical orchids in the world’s richest plant biodiversity hotspot. – New Phytol. 215: 891–905.

Pinkert, R. 1965. Interessante und neue Funde und Erkenntnisse für die Lepidopterenfauna der Kanaren III. – Zeitschrift der Wiener Entomologischen Gesellschaft 50: 153–167, pls 119–123.

Pridegon, A. et al. 2001. Genera Orchidacearum, Vol. 2: Orchidoideae (Part 1). – Oxford Univ. Press.

Ratnasingham, S. and Hebert, P. D. 2007. BOLD: the barcode of life data system (<www.barcodinglife.org>). – Mol. Ecol. Notes 7: 355–364.

Ratnasingham, S. and Hebert, P. D. 2013. A DNA-based registry for all animal species: the barcode index number (BIN) system. – PLoS One 8: e66213.

Reyes-Betancourt, J. A. et al. 2008. Diversity, rarity and the evolution and conservation of the Canary Islands endemic flora. – Anales Jard. Bot. Madrid, pp. 25–45.

Seguí, J. et al. 2017. Species–environment interactions changed by introduced herbivores in an oceanic high-mountain ecosystem. – AoB Plants 9, doi: 10.1093/aobpla/plw084.

Senghas, K. 1992. *Habenaria*. – In: Schlechter, R. et al. (eds), Die Orchideen: ihre Beschreibung, Kultur und Züchtung, Erster Band. Teil A, Botanische Grundlagen der Orchideenforschung, Taxonomischer Teil. Parey, p. 1128.

Singer, R. et al. 2007. The pollination mechanism of *Habenaria pleiophylla* Hoehne & Schlechter (Orchidaceae: Orchidinae). – Funct. Ecosyst. Commun. 1: 10–14.

Singer, R. B. 2001. Pollination biology of *Habenaria parviflora* (Orchidaceae: Habenariinae) in southeastern Brazil. – Darwiniana 39: 201–207.

Sletvold, N. and Ågren, J. 2010. Pollinator-mediated selection on floral display and spur length in the orchid *Gymnadenia conopsea*. – Int. J. Plant Sci. 171: 999–1009.

Solana, M. and Aparicio, A. 1999. Reconstruction of the 1706 Montaña Negra eruption. Emergency procedures for Garachico and El Tanque, Tenerife, Canary Islands. – Geol. Soc. Lond. Spec. Publ. 161: 209–216.

Steinbauer, M. J. and Beierkuhnlein, C. 2010. Characteristic pattern of species diversity on the Canary Islands. – Erdkunde 64: 57–71.

Stierstorfer, C. and von Gaisberg, M. 2006. Annotated checklist and distribution of the vascular plants of El Hierro, Canary Islands, Spain. – Englera 27: 3–221.

Subedi, A. et al. 2011. Pollination and protection against herbivory of Nepalese Coelogyninae (Orchidaceae). – Am. J. Bot. 98: 1095–1103.

Suetusgu, K. and Tanaka, K. 2014. Diurnal butterfly pollination in the orchid *H. abenaria radiata*. – Entomol. Sci. 17: 443–445.

Szlachetko, D. L. and Rutkowski, P. 2000. *Gymnostema orchidarium* I. – Acta Bot. Fenn. 169: 1–380.

Tahalaj, I. and Brzosko, E. 2008. Selfing potential in *Epipactis palustris*, *E. helleborine* and *E. atrorubens* (Orchidaceae). – Plant Syst. Evol. 276: 21–29.

Tao, Z.-B. et al. 2018. Nocturnal hawkmoth and noctuid moth pollination of *Habenaria limprichtii* (Orchidaceae) in sub-alpine meadows of the Yulong Snow Mountain (Yunnan, China). – Bot. J. Linn. Soc. 187: 483–498.

Van der Pijl, L. and Dodson, C. H. 1966. Orchid flowers: their pollination and evolution. – Fairchild Tropical Garden and the Univ. of Miami Press.

Vives Moreno, A. 2014. Catálogo sistemático y sinonímico de los Lepidoptera de la Península Ibérica, de Ceuta, de Melilla y de las Islas Azores, Baleares, Madeira y Salvajes (Insecta: Lepidoptera).

Waterthall, L. 1997. The pollinators of the Malagasy star orchids *Angraecum sesquipedale*, *A. sororium* and *A. compactum* and the evolution of extremely long spurs by pollinator shift. – Bot. Acta 110: 343–359.

Waterthall, L. T. 1998. Deep flowers for long tongues. – Trends Ecol. Evol. 13: 459–460.

Whittall, J. B. and Hodges, S. A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. – Nature 447: 706–709.

Witt, T. J. et al. 2011. Subfamilia Arctiinae. – In: Witt, T. and Ronkay, L. (eds), Noctuidae Europaeae, volume 13. Lymantriinae. – Acta 447: 706–709.

Xiong, Y.-Z. et al. 2015. Mast fruiting in a hawkmoth-pollinated orchid *Habenaria glaucafilia*: an 8-year survey. – J. Plant Ecol. 8: 136–141.

Supplementary material (available online as Appendix njb-02401 at <www.nordicjbotany.org/appendix/njb-02401>). Appendix 1.