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Research

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, Godfery and Godfery 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 (Taťalaj 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



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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., *Platanthera* 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 Ågren 2009, Sletvold and Ågren 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, Pridgeon 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 (Fig. 1) are situated between 27°37' and 29°25'N and 13°20' and 18°10'W; its easternmost island Fuerteventura 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.

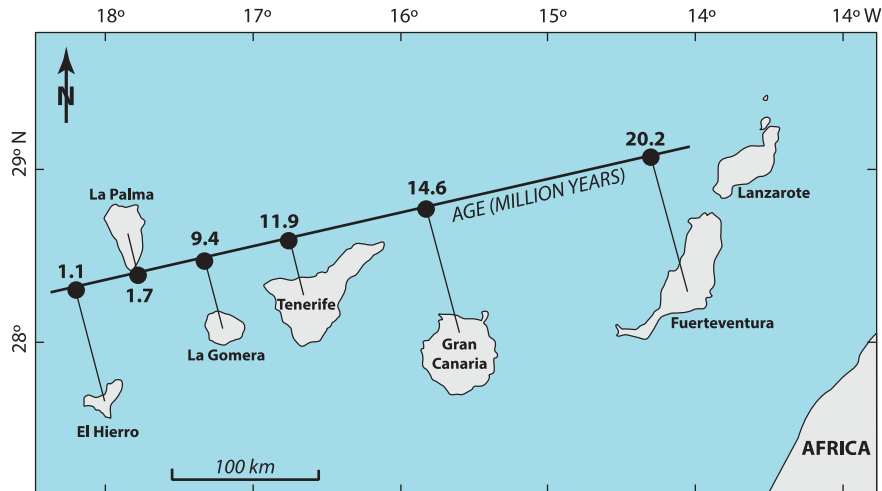


Figure 1. The Canary Islands with their respective geological age. Illustration by Erik-Jan Bosch, modified from Carracedo and Perez-Torrado 2013.

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 Tanque (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 pluviaseasonal thermomediterranean bioclimatic zone under the influence of trade-wind clouds. Vegetation cover was sparse, especially in sites 1, 2 and 3 (El Tanque)

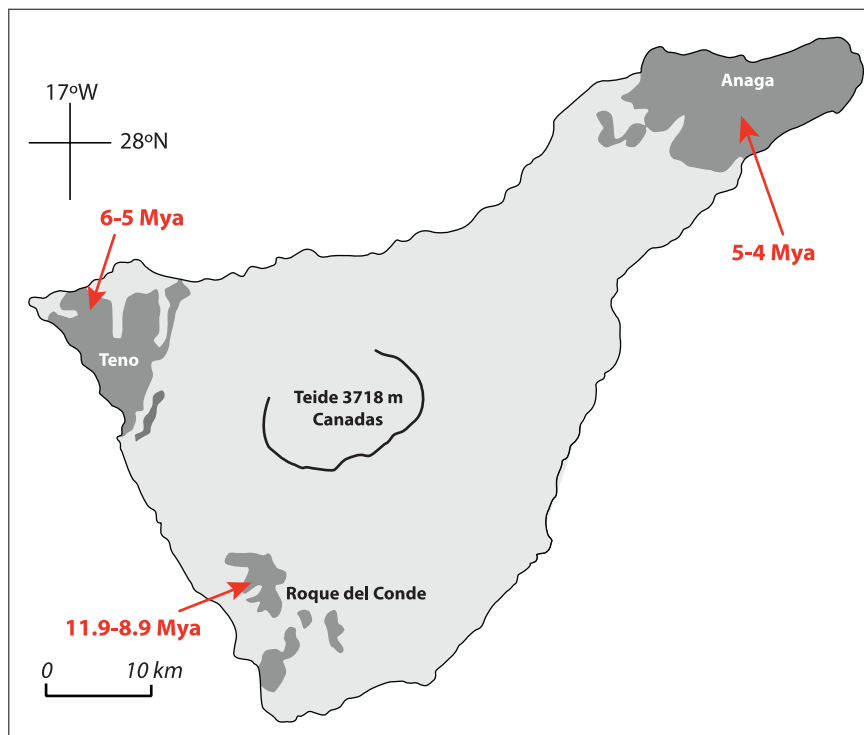


Figure 2. Different parts of Tenerife. The oldest parts Anaga, Teno and Roque del Conde are referred to as Tenerife old, the rest is referred to as Tenerife young. Illustration by Erik-Jan Bosch, modified from Dóniz-Páez et al. 2012.

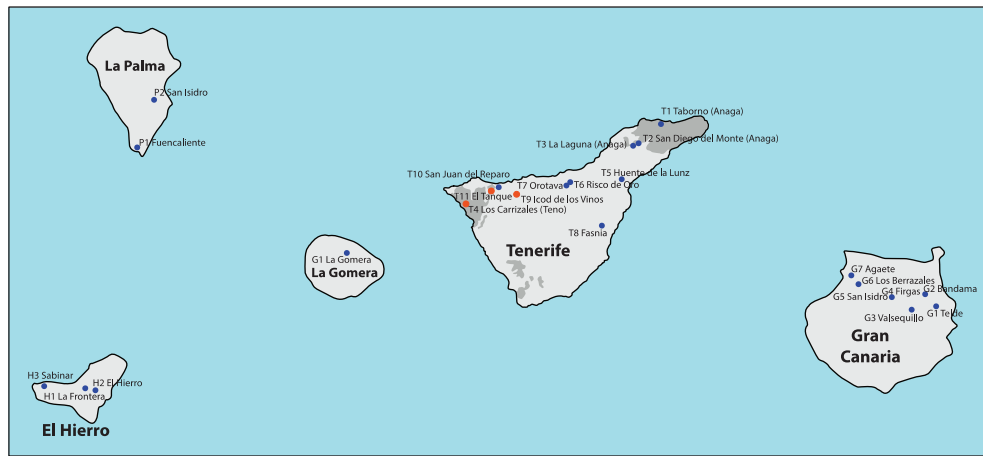


Figure 3. Localities on Canary Islands of which spur lengths of *H. tridactylites* were measured indicated in blue. In addition, pollination was studied in the field in the sites indicated in red. Illustration by Erik-Jan Bosch.

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, odorous 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 Telde	Gran Canaria	-15.425049	27.999277	H	SL
G2 Bandama	Gran Canaria	-15.459113	28.035493	F	SL
G3 Valsequillo	Gran Canaria	-15.501214	27.990528	F	SL
G4 Firgas	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 Agaete	Gran Canaria	-15.684021	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 Hiente 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 ¹	La Gomera	-17.213361	28.164907	H	SL
P1 Fuencaliente	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 ¹	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 (32h) and 21 evenings (73.5h) 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 μ A; exposure time 4–7 s; pictures per sample 1001–1601; camera binning 2; optical magnification 4 $\times$, with a pixel size of 1.4–4.7 μ m. 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 complemented with sequences from published studies in Orchidinae (Inda et al. 2012, Batista et al. 2013). *Disa uniflora* P.J.Bergius was used as outgroup.

DNA extraction, amplification, sequencing and alignments

Total genomic DNA was extracted from about 100 mg of silica gel dried leaf tissue following the 2 $\times$ CTAB (hexadecyltrimethylammonium bromide) protocol for isolating DNA (Doyle and Doyle 1987). We used the 17SE and 26SE primers for amplification of nrITS and 2.1aF and 5R primers for amplification of the plastid *matK* region.

The polymerase chain reaction (PCR) mixture and amplification profiles followed Inda et al. (2012) and Kisel et al. (2012) and sanger sequencing was conducted by BaseClear (<www.baseclear.com>) on an ABI 3730xl genetic analyzer. Newly generated sequences were deposited in NCBI GenBank (Table 1, Supplementary material Appendix 1 Table A8). We used Geneious R9 (Biomatters Ltd., Kearse et al. 2012) for the editing of chromatograms and alignment of sequences.

Phylogenetic analyses and molecular clock analysis

We analyzed the individual and concatenated datasets of the two molecular markers nrITS and *matK* with maximum likelihood (ML) in the CIPRES Science Gateway ver. 3.1 (<www.phylo.org/sub_sections/portal/>) (Miller et al. 2010). The incongruence between plastid and nuclear datasets was assessed with the pipeline implemented by Pérez-Escobar et al. (2017) using the procrustean approach to cophylogeny (PACo) application (Balbuena et al. 2013) in R (<<http://data-dryad.org/review?doi=doi:10.5061/dryad.q6s1f>>). The *matK* sequences from the conflicting terminals were removed in the concatenated dataset (Pérez-Escobar et al. 2016). The new concatenated matrix was re-aligned and used as input to calculate divergence times in *Habenaria* and close relatives. The divergence times were estimated in BEAST ver. 1.8.2 using the CIPRES Science Gateway (Miller et al. 2010) with GTR+G substitution model and four gamma categories, lognormal relaxed uncorrelated clock and tree prior Yule process (Y) model. We used the age estimates from a fossil-calibrated chronogram of Orchidaceae by Pérez-Escobar et al. (2017) in order to perform secondary calibrations. We assigned a normal prior distribution of 43.07 ($\pm$ 2.5 SD) Mya to the root node of *Disa* P.J.Bergius + *Habenaria* and close relatives and 33.78 ($\pm$ 2.0 SD) Mya to the node of divergence of *Disa* and *Habenaria* and close relatives. 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 with 60 $\times$ 10⁶ 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	<i>Eudonia angustea</i> (Curtis, 1827)	4+	yes	S, SW Europe	Los Carrizales; El Tanque	RMNH.5009039 RMNH.5011624 RMNH.5011625 RMNH.5011626 Bacallado s.n. 20131204_DSC9368	–	MK566751
Crambidae	<i>Eudonia lineola</i> (Curtis, 1827)	1	yes	S, SW Europe	El Tanque			
Crambidae	<i>Evergestia isatidalis</i> (Duponchel, 1833)	3	yes	S Europe	Los Carrizales	RMNH.5011610; RMNH.5011611; RMNH.5011627	BOLD:ADL3576	MK566741 MK566743 MK566744
Erebidae	<i>Eilema albicosta witti</i> Kobes, 1993	2	yes	Endemic Tenerife	Los Carrizales; Icod de los Vinos	RMNH.5011612; RMNH.5011618	BOLD:ACD0672	MK566750 MK566746
Erebidae	<i>Schrankia costaestrigalis</i> (Stephens, 1834)	2	no	Europe	El Tanque	RMNH.5011617; RMNH.5011622	BOLD:AAD1543	MK566756 MK566754
Geometridae	<i>Ascotis fortunata</i> (Blachier, 1887)	1	yes	Macaronesia	Icod de los Vinos	Bacallado s.n.	–	
Geometridae	<i>Charissa canariensis canariensis</i> (Rebel, 1911)	1	yes	Endemic Canary Islands	Los Carrizales	RMNH.5011609	BOLD:ADL4101	MK566753
Geometridae	<i>Costaconvexa centrostrigaria</i> (Wollaston, 1858)	1	yes	Macaronesia, N America	Icod de los Vinos	Bacallado s.n.	–	
Geometridae	<i>Cyclophora maderensis trilineata</i> (Prout, 1934)	34	yes	Endemic Canary Islands	Icod de los Vinos	Bacallado s.n.	–	
Geometridae	<i>Episauris kiliani</i> (Rebel, 1898)	2	yes	Endemic Canary Islands	Icod de los Vinos	Bacallado s.n.	–	
Geometridae	<i>Gymnoscelis rufifasciata</i> (Haworth, 1809)	1	no	Europe	El Tanque	RMNH.5011616	BOLD:ADL3671	MK566745
Geometridae	<i>Nebula ibericata</i> (Staudinger, 1871)	3	yes	SW Europe	El Tanque	RMNH.5009041 RMNH.5009042 RMNH.5011615	BOLD:ACF0717	MK566759
Noctuidae	<i>Cucullia calendulae</i> (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	<i>Paranataelia whitei</i> (Rebel, 1906)	1	no	Endemic Canary Islands	Los Carrizales	RMNH.5011608	BOLD:ADL3062	MK566758
Tortricidae	<i>Clepsis coriakanus</i> (Rebel, 1894)	1	no	Endemic Canary Islands	Los Carrizales	RMNH.5011613	BOLD:ADL2652	MK566740
Tortricidae	<i>Acroclita sonchana</i> Walsingham, 1908	3	yes	Endemic Canary Islands	Los Carrizales; El Tanque	RMNH.5011614; RMNH.5011621; RMNH.5011623	BOLD:ADL4602	MK566747 MK566757 MK566748

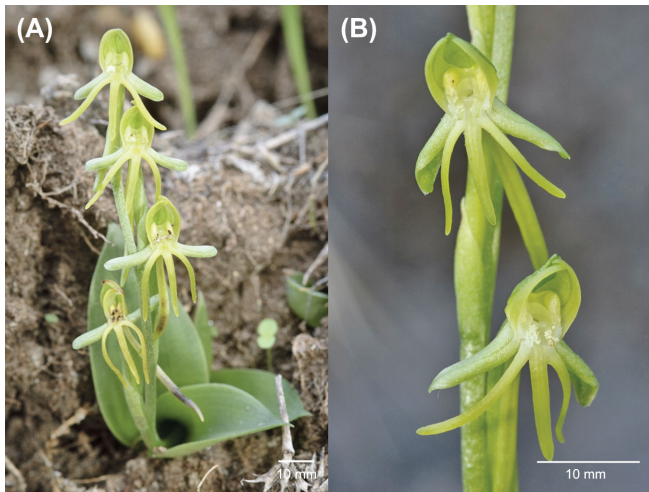


Figure 4A–B. (A) Habitats of *H. tridactylites*. (B) Close-up of part of the inflorescence of *H. tridactylites*. Photographs by Jean Claessens on Tenerife, El Amparo, on 01.01.2008. Scale bar = 10 mm.

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



Figure 4C. Longitudinal section of a flower of *H. tridactylites*. A=anther, RA=rostellar arm, T=tongue-shaped elevation on the lip base, V=viscidium, SL=stigmatic lobe, S=spur, O=ovary. Photograph by Jean Claessens. Scale bar = 1 mm.



Figure 4D. Biotope of *H. tridactylites* with *Pinus canariensis* and *Davallia canariensis*. Photograph by Jean Claessens at El Tanque on 19 Nov 2014. Scale bar = 10 mm.

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

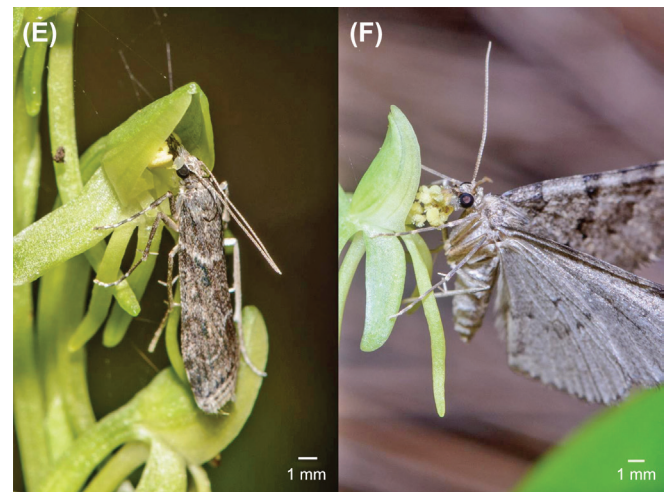


Figure 4E–F. (E) *Eudonia lineola* pollinating *H. tridactylites* with a viscidium sticking to its eyes at El Tanque on 8 Dec 2013. (F) *Nebula ibericata numidata* pollinating *H. tridactylites* with a bunch of pollinaria sticking to the base of the proboscis at El Tanque on 4 Dec 2013. Photographs by Jean Claessens. Scale bar = 1 mm.



Figure 4G. *Cyclophora maderensis trilineata* carrying pollinaria of *H. tridactylites* at Tenerife, El Tanque on 15 Jan 2016. The viscidia stick to the eyes. Photograph by Jean Claessens. Scale bar = 1 mm.

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 *Cyclophora 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



Figure 4H. *Cucullia calendulae* pollinating *H. tridactylites* at Tenerife on Los Carrizales on 2 Dec 2014. Photograph by Jean Claessens. Scale bar = 10 mm.

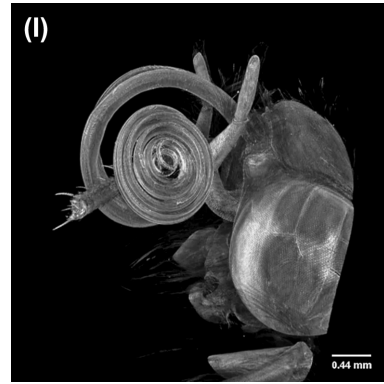


Figure 4I. CT scan of the proboscis of RMNH5011606. Photograph by Rob Langelaan. Scale bar = 0.44 mm.

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

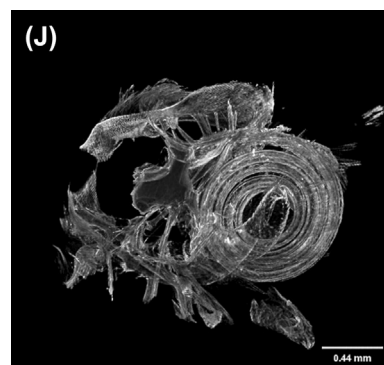


Figure 4J. CT scan of RMNH5011610. Photograph by Rob Langelaan. Scale bar = 0.44 mm.

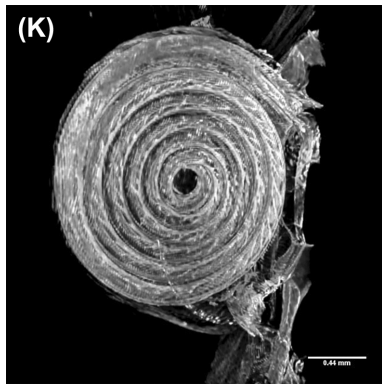


Figure 4K. CT scan of RMNH5011611. Photograph by Rob Langelaan. Scale bar = 0.44 mm.

identification numbers (BIN's) for the endemic *Paranataelia whitei* Rebel, *Charissa canariensis canariensis* Rebel, *Acroclita sonchana* Walsingham and *Clepsis coriacanus* 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 calendulae* 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 $ca\ 18.5 \pm 2.5$ Mya (Supplementary

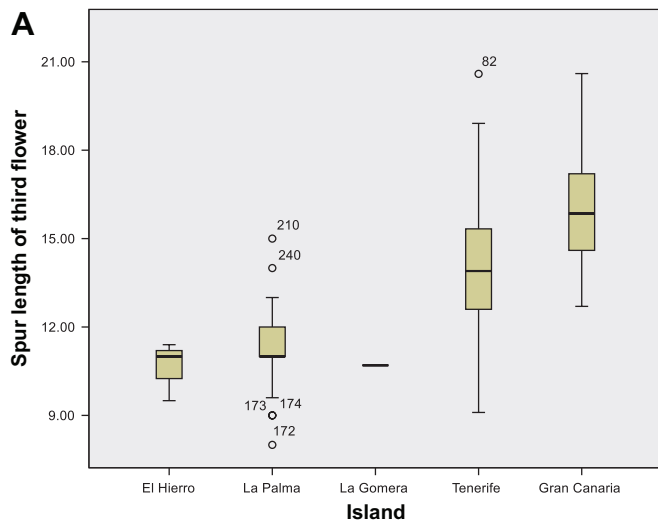


Figure 5A. Boxplot of spur length (mm) of *H. tridactylites* per island.

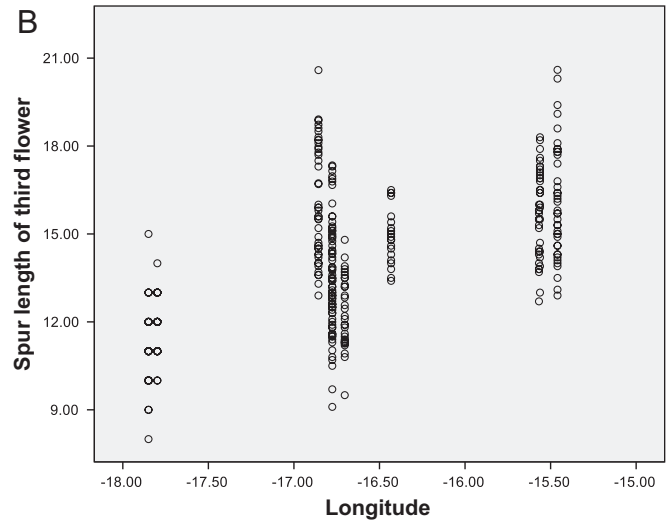


Figure 5B. Scatterplot of spur length (mm) of *H. tridactylites* against distance to mainland Africa (measured by longitude).

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

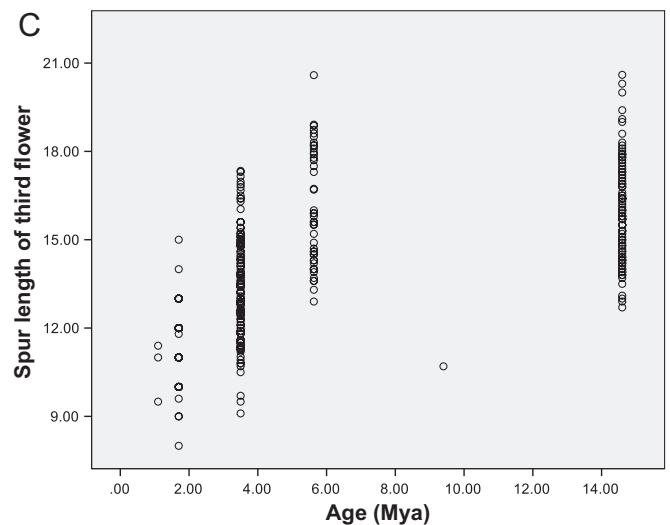


Figure 5C. Scatterplot of spur length (mm) of *H. tridactylites* against mean geological age of the locality of the plant in the field (measured in million years ago).

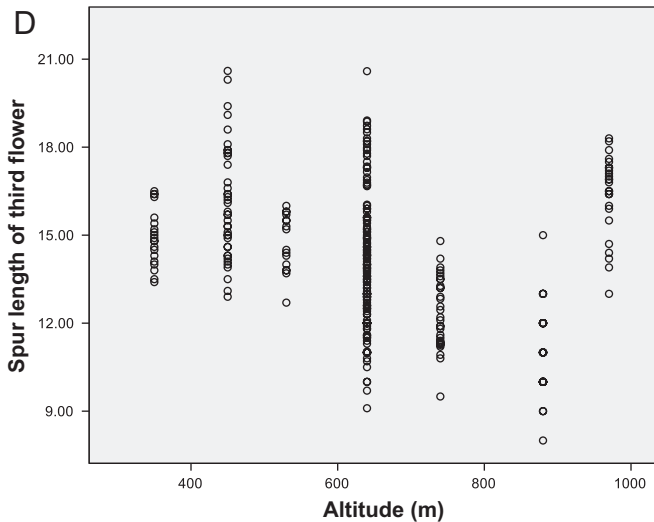


Figure 5D. Scatterplot of spur length against altitude.

2014) 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 another. 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

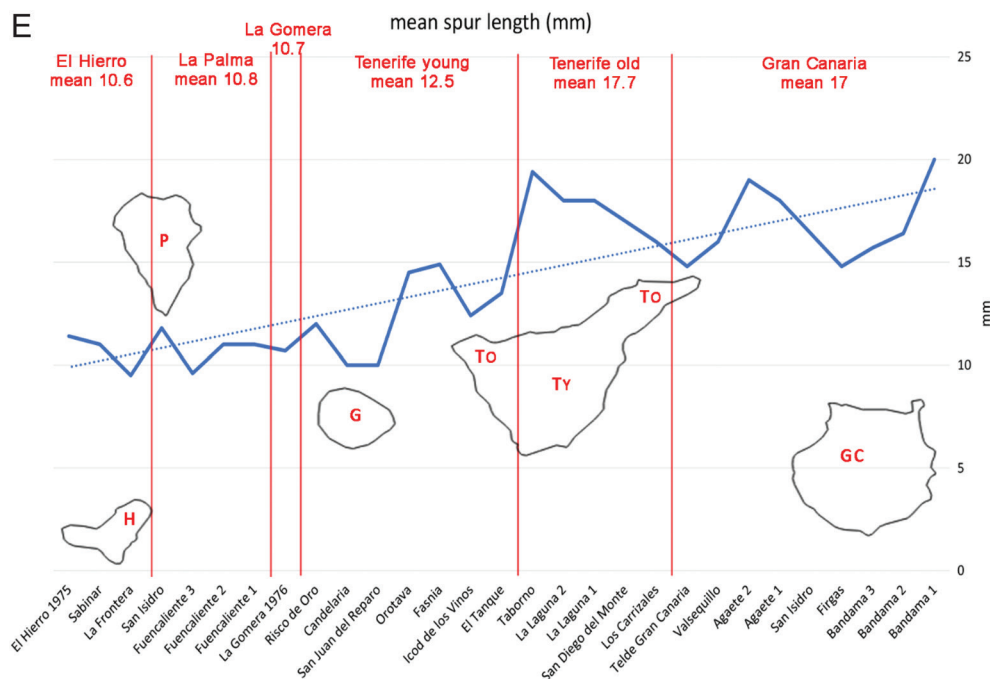


Figure 5E. Mean spur length of *Habenaria tridactylites* on the Canary Islands.

tree heath, *Erica arborea* (Fayal-brezal 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-brezal (*Myrica fayae*–*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 ($R^2 = 36\text{--}38\%$, $p < 0.000$). In both cases, the regression coefficient is significantly positive. A multiple regression with both explanatory variables (age and longitude) also gives a significant model ($R^2 = 38\%$, $p < 0.000$) (Supplementary material Appendix 1 Table A7). However, there is a multicollinearity problem, as the explanatory variables age and longitude are correlated. This discredits the significance calculations of the coefficients in a multiple regression; and for that reason, we preferred to address separate simple regressions.

Mean spur length was the highest in the oldest island, Gran Canaria and showed a decrease from east to west, that is from the oldest to the youngest islands, La Palma and El Hierro (Fig. 5D). Tenerife showed a large variation in spur length, but the longest spurs were found on the oldest parts of the island, Anaga and Teno, two of the shield volcanoes that were eventually merged by the eruption and subsequent emergence of the column of the Teide volcano. A shorter spur length was found in younger parts, which were colonised only after the formation of the Teide. The pollinator shift theory (Wasserthal 1997, 1998, Whittall and Hodges 2007, Hodges and Whittall 2008) assumes that saltatory mutations

driving the length of the spur evolve as an adaption to the tongue length of a new pollinator guild. This theory seems not applicable to *H. tridactylites* on the Canary Islands, since there is only one pollinator guild (Lepidoptera) and the flowers are attractive to Lepidoptera with various tongue lengths. The spur length does not show discrete length differences between the different islands or elevation either, but a gradual elongation from the youngest to the oldest islands instead. The increasing mean spur length on older islands might be in accordance with the evolutionary arms race theory as first pointed out by Darwin (1877). According to this theory, proboscis length and spur length are reciprocally influenced by positive feedback, leading to a constantly growing elongation of both. This idea was later on elaborated by various other authors (Nilsson 1988, 1998, Whittall and Hodges 2007, Anderson and Johnson 2008, Pauw et al. 2009). According to this theory, spur length is age-dependent and could thus explain the distribution of mean spur length of *H. tridactylites* on the various Canary Islands as confirmed in this study.

We only collected data on pollinator tongue length from three regions of a single island, Tenerife (El Tanque, Icod de los Vinos and Los Carrizales) so more data on local pollinator tongue lengths are needed from the other Canary Islands with contrasting geological ages. The shortest spurs of *H. tridactylites* on El Hierro and La Palma are probably related to the fact that these are geologically the youngest Canary Islands and that these islands are also separated from the mainland by the greatest distance. When comparing island specimens of butterflies to mainland specimens of the same species, the individuals of island populations tend to be smaller, especially for small, weak fliers that cannot easily make the crossing (Garth and Tilden 1986). It might be that the mean tongue length of the pollinators of *H. tridactylites* on El Hierro and La Palma is shorter due to this so-called island effect. More experimental data are needed from common garden studies to further investigate this.

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.

Analysing the free spur space (FSS) seems to be more realistic than measuring spur length, because this determines whether a potential pollinator can reach nectar accumulated in the spur. Our analyses showed that this measure is quite flexible, enabling even insects with a short proboscis to consume at least some nectar. Combined with the general poverty of flowering plants during the flowering period of

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