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Pollen foraging behaviour of solitary Hawaiian bees revealed through molecular pollen analysis

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Abstract

Obtaining quantitative information concerning pollinator behaviour has become a primary objective of pollination studies, but methodological limitations hinder progress towards this goal. Here, we use molecular genetic methods in an ecological context to demonstrate that endemic Hawaiian *Hylaeus* bees (Hymenoptera: Colletidae) selectively collect pollen from native plant species in Haleakala and Hawaii Volcanoes National Parks. We identified pollen DNA from the crops (internal storage organs) of 21 *Hylaeus* specimens stored in ethanol for up to 3 years. Genetic analyses reveal high fidelity in pollen foraging despite the availability of pollen from multiple plant species present at each study site. At high elevations in Haleakala, pollen was available from more than 12 species of flowering plants, but Hawaiian silversword (*Argyroxiphium sandwicense* subsp. *macrocephalum*) comprised 86% of all pollen samples removed from bee crops. At lower elevations in both parks, we only detected pukiawe (*Leptecophylla* (*Styphelia*) *tameiameiae*) pollen in *Hylaeus* crops despite the presence of other plant species in flower during our study. Furthermore, 100% of *Hylaeus* crops from which we successfully identified pollen contained native plant pollen. The molecular approaches developed in this study provide species-level information about floral visitation of Hawaiian *Hylaeus* that does not require specialized palynological expertise needed for high-throughput visual pollen identification. Building upon this approach, future studies can thus develop appropriate and customized criteria for assessing mixed pollen loads from a broader range of sources and from other global regions.

Keywords: floral fidelity, foraging ecology, *Hylaeus*, native pollinators, pollination, silversword

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Introduction

Pollination of flowering plants is an essential ecosystem service (Kremen *et al.* 2007; Lonsdorf *et al.* 2009), key aspects of which hinge on the foraging behaviour of pollinators (Schemske & Horvitz 1984; Wilson & Thomson 1991; Dick *et al.* 2003). Traditional approaches to characterizing relationships between plants and pollinators rely on time-intensive observations of individual interactions (Mitchell *et al.* 2009). Because observations of floral visitation may not reflect pollinator efficiency or the species identity of collected pollen (Schemske & Horvitz 1984; Muchhala *et al.* 2009), definitive descriptions of plant–pollinator interactions require the direct identification of pollen carried by pollinators. Studies examining insect-collected pollen often visually compare field-collected pollen samples to voucher specimens in pollen libraries; such efforts employ specialists experienced in pollen identification (e.g. Beattie 1971; Scott 1996; Horskins & Turner 1999; Williams & Kremen 2007; Muchhala *et al.* 2009). Molecular approaches for identifying field-collected pollen, however, are under-utilized in pollinator ecology even though plant geneticists routinely analyse leaf tissue and pollen samples using PCR-based methods (Petersen *et al.* 1996; Parducci *et al.* 2005; Chen *et al.* 2008; Brunet & Holmquist 2009).

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In this study, we use DNA barcoding to identify pollen from the crops (internal storage organs) of endemic Hawaiian *Hylaeus* bees (Hymenoptera: Colletidae) collected several years prior to pollen analysis. A cosmopolitan genus, *Hylaeus*, represents the only native bee group to have colonized the Hawaiian Islands, where it underwent an extensive radiation that resulted in a single clade of 60 endemic species (Daly & Magnacca 2003; Michener 2007). Despite recent advances in the systematics and biogeography of Hawaiian *Hylaeus* (Magnacca 2005; Magnacca & Danforth 2006, 2007), their precise ecological importance remains largely unknown (Daly & Magnacca 2003; Magnacca 2007). As the only native bees in this region, they are believed to serve as important pollinators of many Hawaiian plants, including the endangered Hawaiian silversword (*Argyroxiphium sandwicense*) (Forsyth 2003). Small in size and mostly hairless, *Hylaeus* species lack the structures for external pollen transport found in most bee species (Michener 2007) and instead transport pollen and nectar inside the crop while foraging (Daly & Magnacca 2003). Gaps in understanding of *Hylaeus* ecology may be due in part to their small size, solitary lifestyle and the difficulty in distinguishing among species in the field.

Because little is known about *Hylaeus* ecology beyond floral visitation records (Daly & Magnacca 2003), we conducted a molecular examination of crop contents for bee specimens originally collected for use in other studies. Our approach provides information about floral resource exploitation that supplements observational studies conducted in this system. Future studies on rare or locally distributed pollinators (e.g., like some *Hylaeus* species) could benefit from this approach because no additional sampling is required to analyse pollen present in previously collected specimens. Molecular pollen analysis can also yield information concerning pollen use and niche breadth, thus advancing a general understanding of plant–insect interactions.

Materials and methods

We sampled *Hylaeus* from two national parks: Haleakala National Park (HALE) on the island of Maui and Hawaii Volcanoes National Park (HAVO) on the big island of Hawaii. Study sites were located in open *Metrosideros polymorpha* (ohia) woodlands between 1000 and 1200 m (HAVO), subalpine shrublands between 2200 and 2500 m (HALE) and high-elevation cinder habitat between 2800 and 3000 m (HALE). We hand-netted *Hylaeus* in early summer (2006) and late spring (2007 and 2008) in HAVO and HALE. For collected bees, we noted the plant species or substrate from which individuals were captured. The composition of flowering plant communities differed among these collection sites, as determined by a general survey of the study sites (Table 1).

Identification of bees

After collection, we preserved bees in either 70% ethanol ($N = 21$; samples from 2007) or 100% ethanol ($N = 40$; samples from 2006 and 2008) and stored samples at room temperature. We identified 61 *Hylaeus* specimens to species by sequencing the cytochrome oxidase I gene using primers from Magnacca & Danforth (2006). We extracted bee DNA from thoraces using QIAamp DNA Micro Kits (Qiagen). For PCR, we used the protocol of Wilson *et al.* (2009) with the following reaction conditions: an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at a gradient of 48–53.2 °C for 45 s and extension at 72 °C for 60 s. A final extension step was performed at 72 °C for 10 min.

Identification of pollen

We examined the crop of each bee for pollen through abdominal dissection under a light microscope (Nikon SMZ645). After bisecting full crops, we removed pollen from each half separately. Pollen was first exposed to 5 μL cyclohexane for 20 min to dissolve the protein coat (Doughty *et al.* 1993) and then crushed in liquid nitrogen. We extracted DNA using DNeasy Plant Mini Kits (Qiagen). Pollen samples from bee specimens stored in 100% ethanol were PCR-amplified using primers for the nuclear-encoded internal transcribed spacer regions (ITS) and the 5.8S rDNA region using primers described by Little *et al.* (2004). For pollen samples from bee specimens stored in 70% ethanol, we amplified the 26S (28S) rRNA gene using the primers described by Cullings (1992).

PCRs for pollen were performed in a 15-μL volume on Eppendorf Mastercycler gradient thermal cyclers. We used 3 μL of template DNA, 10X Taq Buffer with 1.5 mM $MgCl_2$ (Eppendorf), 1.5 μL of 1.5 mM $MgCl_2$, 1.5 μL of 10X BSA, 0.3 μL of 10 μM dNTPs, 0.6 μL of each 10 μM primer and 0.15 U of Taq DNA Polymerase (Eppendorf). Reaction conditions for amplifying ITS regions were run as follows: an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at gradient of 48–53.2 °C for 30 s and extension at 72 °C for 45 s. A final extension step was performed at 72 °C for 10 min. To amplify the 28S rRNA gene, we used the conditions described in Cullings (1992) and an annealing temperature of 53 °C. All PCR amplification products were visualized on agarose gels.

Table 1 Plant species in flower at the time of *Hylaeus* collection in Haleakala (HALE) and Hawaii Volcanoes (HAVO) National Parks. An asterisk indicates that the species was present, but relatively rare

Flowering plant species	Status	Site
Argyroxiphium sandwicense subsp. *macrocephalum* (Asteraceae)	Endemic perennial, threatened	Haleakala Crater (HALE)
Gnaphalium sandwicensium (Asteraceae)	Endemic perennial	Haleakala Crater (HALE)
Hypochaeris radicata (Asteraceae)	Non-native perennial	Haleakala Crater (HALE) Halemau'u Trail (HALE)
Plantago lanceolata (Plantaginaceae)	Non-native perennial	Haleakala Crater (HALE)
Rumex acetosella (Polygonaceae)	Non-native perennial	Haleakala Crater (HALE)
Tetramolopium humile (Asteraceae)	Non-native perennial	Haleakala Crater (HALE)
Oenothera stricta (Onagraceae)	Non-native annual	Haleakala Crater (HALE)
Leptecophylla (Styphelia) tameiameiae (Ericaceae)	Endemic perennial	Haleakala Crater (HALE) Halemau'u Trail (HALE) Hilina Pali Road (HAVO)
Vaccinium reticulatum (Ericaceae)	Endemic perennial	Haleakala Crater (HALE) Halemau'u Trail (HALE) Hilina Pali Road (HAVO)
Dubautia menziesii (Asteraceae)	Endemic perennial	Haleakala Crater (HALE) Halemau'u Trail (HALE) Hilina Pali Road (HAVO)
Coprosma ernodeoides (Rubiaceae)	Endemic perennial	Haleakala Crater* (HALE) Halemau'u Trail (HALE)
C. montana (Rubiaceae)	Endemic perennial	Haleakala Crater* (HALE) Halemau'u Trail (HALE)
Geranium cuneatum (Geraniaceae)	Endemic perennial	Halemau'u Trail (HALE)
Sophora chrysophylla (Fabaceae)	Endemic perennial	Halemau'u Trail (HALE) Hilina Pali Road* (HAVO)
Santalum haleakalae (Santalaceae)	Endemic perennial	Halemau'u Trail (HALE)
Metrosideros polymorpha (Myrtaceae)	Endemic perennial	Hilina Pali Road (HAVO)

To purify all PCR products, we used 0.08 μL Exonuclease I and 0.4 μL Shrimp Alkaline Phosphatase (USB Corporation) in a total volume of 5 μL. Samples were incubated at 37 °C for 15 min then heated to 80 °C for 15 min. Purified PCR products were then sequenced (Retrogen, San Diego, CA). We edited and aligned resulting sequences by eye using SEQUENCHER v. 4.10.1 (Gene Codes); any ambiguous peaks were scored as 'N'. Voucher pollen samples were collected from common perennial plants in HALE and HAVO (Table 2); we extracted, amplified and sequenced these samples as previously described for crop pollen.

Because crop samples may contain pollen from multiple plant sources, we determined the level of pollen purity sufficient for successful identification by sequencing known mixes of pollen types from our sites (see Appendix S1, Table S2 in the supporting information). This mixed pollen analysis indicated that sample

Table 2 GenBank accession numbers of plant vouchers collected from Haleakala (HALE) and Hawaii Volcanoes (HAVO) National Parks

Plant species	Family	Location	Accession numbers
Argyroxiphium sandwicense subsp. *macrocephalum* (A. Gray) Merat	Asteraceae	HALE	GU256436, GU256435
Coprosma ernodeoides (A. Gray)	Rubiaceae	HALE	GQ885142, GU256426
C. montana Hillebr.	Rubiaceae	HALE	GQ885143, GU256425
Dubautia menziesii (A. Gray) D. D. Keck	Asteraceae	HALE	GU011985, GU256427
Geranium cuneatum Hook	Geraniaceae	HALE	GU011986, GU256428
Hypochaeris radicata L.	Asteraceae	HALE	GU011987, GU256429
Metrosideros polymorpha Gaudich	Myrtaceae	HAVO	GU011988, GU256430
Santalum haleakalae Hillebr.	Santalaceae	HALE	GU011990, GU256431
Sophora chrysophylla (Salisb.) Seem.	Fabaceae	HALE	GU256424, GU256432
Leptecophylla (Styphelia) tameiameiae (Cham. & Schlecht.) C.M.Weiller	Ericaceae	HALE	GU011991, GU256433
Vaccinium reticulatum Sm.	Ericaceae	HALE	GU011989, GU256434

purity corresponded with sequence quality, length and BLAST score (see Appendix S1, Tables S3 and S4 in the supporting information). Thus, BLAST scores ≥1000 and per cent match ≥ 99% were considered putative matches of dominant pollen species. For this reason, crop pollen sequences were initially identified by comparison to sequences of voucher samples (Table 2) and to published sequences (GenBank).

Results

Of the 61 *Hylaeus* samples, molecular identification revealed the presence of four described bee species (Table S1). Most samples were collected from HALE. Samples from the rim of the Haleakala Crater consisted of *H. nivicola* (*N* = 18) and *H. difficilis* (*N* = 3); molecular identifications from this study corroborated identifications based on morphological characters (P. Krushelnycky, *pers. comm.*). *Hylaeus nivicola* (*N* = 35) and *H. volcanicus* (*N* = 1) were identified based on molecular characteristics from Halemau'u Trail, the lower elevation site at HALE. In the samples from HAVO, we identified individuals as *H. volcanicus* (*N* = 2) and *H. laetus* (*N* = 2).

We detected crop pollen in 46% (28/61) of the individual bees examined (Tables 3 and S1) and successfully extracted pollen DNA from 75% (21/28) of these crops. Most samples that failed to amplify (6/7) had been stored in 70% ethanol. Despite the diversity of plant species in flower during collecting periods (Table 1), the crop contents of *Hylaeus* consisted of pollen from three native plants: *Leptecophylla* (*Styphelia*) *tameiameiae* (*N* = 8), *Argyroxiphium sandwicense macrocephalum* (silversword; *N* = 12) and *Dubautia menziesii* (*N* = 1) (Table 3). Almost all bee crops contained pollen from a single plant species; only WPK01 and WPK16 from Haleakala Crater and Hyl S7 from Halemau'u Trail had pollen from more than one plant species in their respective crops (Tables S5 and S6 in the supporting information); however, sequencing identified the dominant pollen species (Table 3). For the Haleakala Crater Rim samples (WPK01-21) collected on flowering silversword (*A. sandwicense macrocephalum*), 86% (12/14) of crop pollen samples were identified as this plant species. For the remaining 14% (2/14) collected at the crater rim, we detected pollen only from two native species (*L. tameiameiae* and *D. menziesii*) although 12 species of flowering plants were available (including four non-native species). All *Hylaeus* collected along the Halemau'u Trail (HALE) (HylC01-F10) had crops containing the numerically dominant *L. tameiameiae* despite the fact that we captured these bees sunning themselves on rocks or visiting other plant species – predominantly *Geranium cuneatum* and *Sophora chrysophylla*. Similarly at

Table 3 Identification of Hylaeus and crop contents for bees with partial and full crop loads. Bees were collected as part of other projects or as voucher specimens. The location and year of collection are listed. Molecular analysis of crop contents revealed information regarding foraging habits. See Table S1 in the Supporting Information for identifications of bees with empty crops. Asterisks indicate samples of mixed origin

Bee ID	Location	Year	Molecular ID of bee	Molecular ID of crop pollen
WPK01	Haleakala crater	2007	*Hylaeus nivicola* (Meade-Waldo)	*Argyroxiphium sandwicense macrocephalum**
WPK02	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK04	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*Leptecophylla (Styphelia) tameiameiae*
WPK06	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK07	Haleakala crater	2007	*H. difficilis* (Perkins)	*Dubautia menziesii*
WPK08	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK09	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK12	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK14	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK15	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK16	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK19	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum**
WPK20	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
WPK21	Haleakala crater	2007	*H. nivicola* (Meade-Waldo)	*A. sandwicense macrocephalum*
Hyl C04	Halemau'u Trail	2008	*H. nivicola* (Meade-Waldo)	*L. tameiameiae*
Hyl C07	Halemau'u Trail	2008	*H. nivicola* (Meade-Waldo)	*L. tameiameiae*
Hyl C08	Halemau'u Trail	2008	*H. nivicola* (Meade-Waldo)	*L. tameiameiae*
Hyl S07	Halemau'u Trail	2008	*H. nivicola* (Meade-Waldo)	*L. tameiameiae**
Hyl S09	Halemau'u Trail	2008	*H. nivicola* (Meade-Waldo)	*L. tameiameiae*
DF01	Hilina Pali Rd	2006	*H. volcanicus* (Perkins)	*L. tameiameiae*
DF02	Hilina Pali Rd	2006	*H. volcanicus* (Perkins)	*L. tameiameiae*

HAVO, all pollen samples were of *L. tameiameiae* ($N = 2$) although bees were only observed visiting *Metrosideros polymorpha*. No pollen DNA was recovered from the one *H. laetus* whose load consisted of a few pollen grains.

Observations of flowers visited by *Hylaeus* did not necessarily reflect the presence or identity of crop pollen in the individuals collected. For example, of the 21 *Hylaeus* captured on silversword, seven individuals carried no crop pollen and two had crops full of nonsilversword pollen (Table 3); these bees likely visited silversword without collecting its pollen and thus may have been consuming nectar at their time of collection. Similarly, no bees from Halemau'u Trail (HALE) and Hilina Pali Road (HAVO) were collected on *L. tameiameiae* although this was the only pollen species ($N = 7$) found in their crops.

For the samples from Haleakala Crater Rim (HALE) (WPK01-21), there were small amounts of pollen (mean ± SE: 27 ± 11 grains) externally visible on bees at the time of collection (P. Krushelnycky, *pers. obs.*). When the crops of these bees were inspected, the number of pollen grains on the surface of the bee body did not predict the presence or absence of pollen in the internal crop (Logistic regression: $X_1^2 = 0.91$, $N = 21$, $P = 0.34$).

Discussion

Through the application of molecular methods to an ecological problem, this study provides a novel perspective on the foraging behaviour of Hawaii's sole group of native bees. We found that Hawaiian *Hylaeus* exhibit high floral fidelity in terms of *pollen collection*: we detected pollen from only a few plant species in our overall sample of bees, and most individual bees consumed pollen from a single plant species. These findings support visual assessments of *H. pubescens* nests in HAVO (Daly & Coville 1982), where pollen provisions appeared to be derived from a single plant species when visually examined. Furthermore, the role of *Hylaeus* as a key pollinator of native plants (perhaps especially silversword) is supported by the detection of only native plant pollen in crop samples. The absence of non-native plant pollen from our samples is consistent with anecdotal evidence that Hawaiian *Hylaeus* rarely visit floral resources of non-native plants (Magnacca 2007).

Given the decline of native pollinators throughout Hawaii (e.g. Hawaiian avifauna) (Stone & Loope 1987; Scott *et al.* 1988), understanding the ecological role of *Hylaeus* and the relationships between these bees and native plants is of central importance to conserve and manage Hawaii's natural resources. As introduced species continue to alter the structure and function of island ecosystems (Vitousek *et al.* 1987), the apparent selectivity of *Hylaeus* for native plants suggests that these bees may face population declines because of reductions in their preferred resources in the face of the introduced plant proliferation.

Our results in part corroborate earlier work by Scott (1996), who reported selective pollen foraging by *Hylaeus* (*H. basalis, H. ellipticus,* and *H. verticalis*) in North America; these species predominantly provisioned their nests with Rosaceae pollen. In comparison, Hawaiian *Hylaeus* species appear to exhibit even more restrictive levels of pollen foraging than mainland species; we detected only three species of pollen from two plant families (Asteraceae and Ericaceae), although species from as many as 10 families were commonly flowering in the collection areas. Findings of selective pollen foraging in New World *Hylaeus* described in this study and by Scott (1996) contrast with floral visitation records from southern Germany (Westrich 1989). Analyses of Westrich's records suggest a wider niche breadth for some continental *Hylaeus* (Waser *et al.* 1996). Given the visitation records of both Hawaiian (Daly & Magnacca 2003) and continental species (Westrich 1989), *Hylaeus* visit more plant taxa than pollen analyses would suggest. Behavioural differences between nectar and pollen foraging likely explain the large discrepancy between floral visitation and pollen records (Scott 1996); bees may need to visit many more plants to collect the nectar resources required to both provision offspring and sustain themselves.

General relevance to plant–pollinator relationships

Our study provides a novel example of how pollen can be identified from pollinator samples collected in the past. Such applications promise to enhance an understanding of plant–pollinator relationships and may provide unexpected details about past plant–pollinator interactions. Moreover, recent DNA barcoding efforts provide an expansive collection of consensus sequences, and thus may facilitate the identification of pollen loads. Given the success of our method at identifying samples not originally preserved for molecular analysis (e.g. in 70% ethanol) and providing information regarding the degree and identities of mixed samples, this approach might be applied in the future to museum specimens and historical pollen samples collected from other insect species. Molecular identification of collected pollen from historical samples (Parducci *et al.* 2005) would be of particular value in biogeographical and conservation studies. This study presents a framework for how common molecular tools can be employed to

assess and identify pollen loads for potentially high-throughput analysis, although the criteria derived for assessing pollen load purity were based on a few species from two families as appropriate for the scope of this study. However, these criteria should not be considered definitive until additional studies have been conducted on samples from other regions that support greater taxonomic diversity of plants compared to our study sites. Future research might also apply our method to corbicular and scopal pollen samples. Using molecular pollen analyses in conjunction with more traditional approaches in this area of study could improve characterizations of floral resource exploitation and contribute to an understanding of how pollinators use plant resources across landscapes. Such information is necessary for conservation and effective management of both natural areas (Chapman *et al.* 2003) and agroecosystems (Weibull *et al.* 2003).

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All authors are actively involved in insect ecology research. EEW's research examines how species interactions and subsequent population-level effects influence food web dynamics. CSS is involved in developing methods to promote native and wild pollinators in agroecosystems. KELV investigates the effects of invasive species on food webs and trophic dynamics using molecular and field-based approaches. DAH is an ecologist studying factors that contribute to the success of invasive species.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 *Hylaeus* and crop content identifications. Bees were collected as part of other projects or as voucher specimens.

Table S2 The composition (identity and proportion) of samples for mixed pollen analysis

Table S3 Mean pollen purity measures and BLAST scores for vouchers and mixed pollen samples

Table S4 Pollen purity analysis using Wilcoxon Signed-Rank test of the 18 mixed pollen samples that yielded BLAST matches

Table S5 Pollen purity assessments for *Hylaeus* pollen loads

Table S6 Ambiguous base locations of crop pollen samples with more than 1 ambiguous base

Appendix S1 Protocols for analyzing mixed samples and deriving pollen purity criteria used for assesssing the identity and number of contributing sources to pollen samples.

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