Viewpoints

Forecasting pollination declines through DNA barcoding: the potential contributions of macroecological and macroevolutionary scales of inquiry

Summary

While pollinators are widely acknowledged as important contributors to seed production in plant communities, we do not yet have a good understanding of the importance of pollinator specialists for this ecosystem service. Determination of the prevalence of pollinator specialists is often hindered by the occurrence of cryptic species and the limitations of observational data on pollinator visitation rates, two areas where DNA barcoding of pollinators and pollen can be useful. Further, the demonstrated adequacy of pollen DNA barcoding from historical records offers opportunities to observe the effects of pollinator loss over longer timescales, and phylogenetic approaches can elucidate the historical rates of extinction of specialist lineages. In this Viewpoint article, we review how advances in DNA barcoding and metabarcoding of plants and pollinators have brought important developments to our understanding of specialization in plant–pollinator interactions. We then put forth several lines of inquiry that we feel are especially promising for providing insight on changes in plant–pollinator interactions over space and time. Obtaining estimates of the effects of reductions in specialists will contribute to forecasting the loss of ecosystem services that will accompany the erosion of plant and pollinator diversity.

Introduction

Specialization in plant–pollinator interactions is a field of study that, like many in biology, is plagued with hidden players and cryptic mechanisms (e.g. pollen is small, and the act of pollen delivery is difficult to evaluate with the human eye; Vamosi *et al.*, 2012). Further, on large geographical scales, the speed and cost of the necessary detailed measurements are often prohibitive. Studies of plant–pollinator interactions require the accurate identification of both pollinators and the pollinated, and recent efforts with DNA barcoding has demonstrated success in achieving this objective without prolonged observations. Landscape-level studies of the effects of changes in pollinator composition on ecosystem health are exceedingly rare (Fründ *et al.*, 2013; Tur *et al.*, 2013) because of the difficulties in getting sample sizes needed to make firm conclusions. Plant–pollinator network studies often rely on comparing a small number of communities (e.g. two), and even these require >100 h of visitor observation (Tur *et al.*, 2013). Estimating the effects of land use on the disappearance of specialists can require a herculean effort, such as the recent study that conducted 962 h of surveying of pollinator visitation in 119 grassland sites with varying levels of disturbance (Weiner *et al.*, 2014), yet such studies will become increasingly necessary if we wish to evaluate the effect of species loss on ecosystem services. Here, we address how DNA barcoding can vastly reduce the effort of these macroecological studies.

The field hours documenting plant–pollinator interactions do not account for the hours spent in the laboratory identifying pollinators, and DNA barcoding is well established to assist with this endeavor (Sheffield *et al.*, 2009). For example, bees and other insects can be identified with great accuracy using the standard animal barcode cytochrome oxidase 1 (CO1) fragment (Hebert *et al.*, 2003b), and a survey of European bees found that results from DNA barcoding largely agreed with traditional taxonomy (Schmidt *et al.*, 2015). Further, other studies reveal that morphologically indistinguishable (i.e. cryptic) species can be differentiated through DNA barcode markers (Smith *et al.*, 2006; Schmidt *et al.*, 2015) or other molecular methods (e.g. the cryptic species group previously known solely as *Halictus ligatus*; Packer *et al.*, 2016) although the converse is also occasionally true (Gibbs, 2010).

Identifying insects through DNA barcoding has become relatively standard in the field, yet DNA barcoding the pollen found on pollinators is a more recent development. The situation is not as simple with plants because at least two gene fragments, typically *rbcL* and *matK*, are required to obtain levels of accuracy above 70% (Pei *et al.*, 2015). Thus, species-level discrimination is more difficult for closely-related species of plants, especially in instances where hybridization occurs (Kress *et al.*, 2005; Clement & Donoghue, 2012). While the use of plastid markers was originally assumed to be inadequate for pollen (thought to be without chloroplasts), several studies have found this not to be the case, expanding the toolbox for pollen DNA barcoding further (reviewed in Bell *et al.*, 2016). With both plants and pollinators, extensive barcode libraries (obtained from herbaria and museum collections) are required to permit ecological samples to be identified. Methodological advances are needed to obtain full species-level resolution when DNA barcoding just the pollen, such as sequencing whole chloroplast genomes or incorporating novel blended approaches between few-marker barcoding and organellar genomics (e.g. Li *et al.*, 2015; Coissac *et al.*, 2016; Hollingsworth

et al., 2016). Nevertheless, recent surveys highlight the rapid development of DNA barcode libraries, with the vast majority of barcodes in BOLD (Ratnasingham & Hebert, 2007; CBOL Plant Working Group, 2009) being for animals (4.30M/406K/154K specimens/BINS/species with publicly available CO1 sequences), followed by plants (301K public specimen records representing 84K species, mainly with *rbcL* sequences but other markers as well; BOLD accessed 26 October, 2016). Here, we explore how these new techniques can expand the scope of previous macroecological investigations on pollination to aid investigations of how specialization evolves and how specialists contribute to ecosystem function.

Metabarcoding is a novel technique that allows multispecies assemblages to be identified from a single sample without separating out individual pollen grains (Box 1). The expanding DNA libraries have been demonstrated capable of reconstructing the plants visited by pollinators simply through DNA barcoding of the pollinator and the pollen on the pollinator's body (Widmer *et al.*, 2000), despite the mixed sample of plant pollen commonly retrieved from polylectic pollinators (reviewed in Bell *et al.*, 2016). Metabarcoding has been used to characterize the composition and relative abundance of pollen collected by honeybees based upon the species of pollen found in scopal loads (Richardson *et al.*, 2015a). These methods have already been used to determine how pollination of a single pollinating species changes with local floristic biodiversity, plant phenology, and the presence of alien flowering species (Wilson *et al.*, 2010; Galimberti *et al.*, 2014), elucidating the potential for macroecological comparisons of pollination over large spatial scales with substantial time savings. A third study identified 19 plant families from honeybee scopal pollen loads and showed that metabarcoding exhibited greater taxonomic sensitivity in large and diverse pollen samples relative to microscopy, which found only eight families (Richardson *et al.*, 2015b), a result that has stood up to scrutiny in other systems that have taken pains to control for the possibility of false positives through trace contaminants (Pornon *et al.*, 2016). Additionally, it has been suggested that metabarcoding is substantially less expensive than traditional barcoding or morphological identification when person hours are included in the costs (Tang *et al.*, 2015). This is especially the case in geographic areas where taxonomic expertise is sparse or for taxonomic groups in which pollen grains are extremely difficult to identify morphologically even to genus level (Schuett & Vamosi, 2010). Of course, in both cases, a locally extensively barcoded fauna/flora is essential to make species-level designations possible and represents a substantial investment.

We argue that investment in these barcode libraries, as well as further development of methods, would provide worthwhile gains for understanding plant–pollinator specialization in ecosystems and outline two areas where recent developments provide windows into future opportunities. First, the costs associated with DNA barcoding and morphological identification are still outside the budgets of most researchers, yet we feel the currently decreasing costs of DNA barcoding will potentially tip the scales towards the methodology should the increased efficiencies continue (Box 1). One recent study was successful at multiplexing 384 pollen samples collected from solitary bees and sequenced all samples together on a single Illumina MiSeq v2 flow cell, revealing 650 different plant taxa visited (of which 617 could be identified taxonomically to plant species level). Because samples were tagged before multiplexing, the suite of plants visited could be determined for each individual sample (Sickel *et al.*, 2015). For comparison, a recent field study that included a similar amount of effort in terms of collecting the samples of pollinators had to limit their surveys to estimate the visitors to only 61 identified plant species (an order of magnitude fewer) through traditional identification of pollinators (Popic *et al.*, 2013). Pantrap samples can also be analyzed with metabarcoding to discover both the suite of pollinators obtained (Tang *et al.*, 2015) as well as the aggregate of pollen they carried with them into the trap.

Because pollinators can visit flowers without contacting the stigmas of plants, what remains to be developed are methods of sampling the composition of pollinators that visited an individual flower directly from trace amounts of pollinator DNA left behind in nectar or on flower surfaces. This type of metabarcoding can be thought of as an environmental DNA (eDNA) approach, which is a promising new method where the identities of a multispecies pool of plant species is recovered from trace amounts of DNA in an environmental sample (Richardson *et al.*, 2015b). For example, there has been success in identifying eDNA of bees from samples of honey (Schnell *et al.*, 2010). Further, some success has been obtained with reconstructing the suite of pollinators of focal plant species from DNA barcoding of 'microbial signatures' in nectar and flower surfaces with that found on particular pollinators. While this practice is still in its infancy and will require further testing (Aizenberg-Gershtein *et al.*, 2013; Ushio *et al.*, 2015), it would circumvent the need for time spent capturing pollinators. Later, we explore how DNA barcoding holds promise to uncover the ecosystem function and conservation importance of pollinator diversity at macroecological scales of inquiry.

DNA barcoding and the potential for understanding plant–pollinator interactions

If we consider the success in developing methods of pollen DNA metabarcoding from sampled pollinators (Bell *et al.*, 2016), these data can be used to construct more rapid estimates of pollinator specialization within communities spanning a large geographical gradient (Box 1). Not only is there the potential for broader sampling, there is the potential for greater accuracy in our estimates of specialization, and these changes should improve our biological inferences regarding mutualisms. For example, DNA barcode markers have demonstrated that many apparent dietary generalist insect species are actually large numbers of specialist cryptic species (e.g. Smith *et al.*, 2011). Similarly, community-level metrics of specialization can change dramatically with DNA barcode data (Clare, 2014; Toju *et al.*, 2014; Roslin & Majaneva, 2016). Generally, there are two sources of errors with traditional identifications as a basis of concluding species interactions: (1) actual misidentifications, especially in large studies with a lot of specimens to identify and (2) cases of missing observations, e.g. due to rare or cryptic species being overlooked. In some systems (e.g. tropics), such studies would not be possible at all without molecular

methods, as the majority of insect species remain undescribed and are certainly unidentifiable in the field (Stork *et al.*, 2015). With DNA barcoding providing valuable information, such as the pollen on a given pollinator species (or Molecular Operational Taxonomic Unit; Galimberti *et al.*, 2014), we are afforded a more accurate estimation of pollinator diet breadth when cryptic species of pollinators are identified (Box 1). Should wide discrepancies be seen between the visually observed visitation patterns and the pollen present on visitors, these methods offer a way of identifying efficient pollinators (Table 1) (Popic *et al.*, 2013).

Identifying pollinator effectiveness, however, will require still more effort in the form of examining whether visitation rates, pollinator identity, and pollen composition on a pollinator's body correspond with conspecific pollen deposition to, and subsequent seed production of, a focal species (Kremen *et al.*, 2002). While the scope of many macroecological questions regarding plant–pollinator interactions can be addressed with DNA barcoding (Box 1), it is important to delineate where and when DNA barcoding approaches will be useful and where they will not. For example, DNA barcoding cannot separate self from nonself pollen: different markers are required for discrimination among individuals of a species (e.g. microsatellites or AFLP). For much of pollination biology involved with the study of inbreeding depression, selfing rates, and the concomitant effects on the evolution of dioecy and floral traits (e.g. inflorescence structure; Lloyd, 1982; Harder & Barrett, 1995), DNA barcoding techniques will be of limited utility. Similarly, understanding specialization of any given species at a population level will also still benefit from traditional ecological approaches because the ability to discern pollen abundance vs presence/absence is still in its infancy (yet there is promise there as well; Tang *et al.*, 2015; Pornon *et al.*, 2016). However, for the effects of pollinator species richness and composition on the sustainability of plant species and communities over macroecological scales, DNA barcoding offers a wealth of opportunities (Table 1). We specifically highlight later several approaches to incorporating barcode information into the study of the ecosystem services provided by specialists in plant–pollinator systems.

Variation in specialization and pollination services

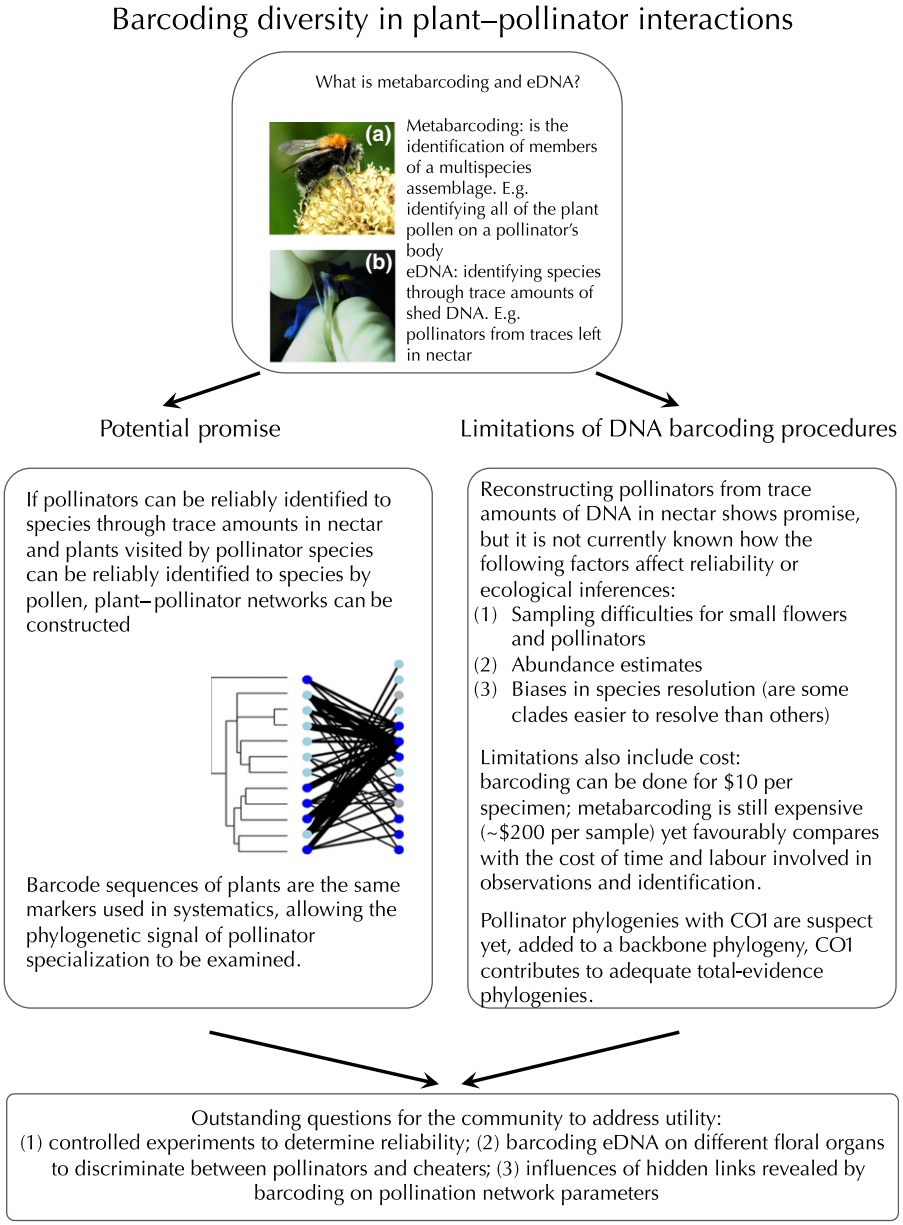
Over macroecological and macroevolutionary scales, the designation of specialization as a species trait is complicated because specialization can change over both space and time, and there are logistical barriers to conducting numerous surveys of plant–pollinator interactions over a large number of sites or years. Nevertheless, even our curtailed sampling to date demonstrates that the extent of specialization observed can depend heavily on how often alternate partners are encountered, which depends on the relative abundance and range overlap with potential mutualists (Sjodin, 2007; Vamosi *et al.*, 2014a). While high pollinator species diversity is often argued to offer benefits in the form of pollination services to plant species (Kremen *et al.*, 2002), the mechanisms underlying this important biodiversity–ecosystem service (BES) relationship are still poorly understood. Due to the ephemeral nature of specialist lineages over space and time, the presence of specialists is often assumed to be of limited importance in the delivery of pollination services (Memmott *et al.*, 2004) and may, therefore, not greatly impact plant reproduction and extinction. However, recent studies that have examined pollen delivery instead of visitation have found that the disappearance of specialists impacts the foraging behavior of the remaining generalists and, in turn, the pollen delivery to the plant community (Brosi & Briggs, 2013). DNA barcoding techniques offer expanded opportunities for the large-scale study of plant–pollinator specialization such that we can: (1) determine how pollinator diversity relates to pollen-delivery adequacy of a plant community; and (2) refine measurements of specialization for both the pollinators and the plants and examine how these change across gradients (Table 1). We describe these two avenues of inquiry below.

Pollinator specialization runs a broad spectrum from extreme specialization of a plant species on a single pollinator species to incorporating upwards of > 100 mutualist species (Vamosi *et al.*, 2013), yet evidence for whether higher species diversity of pollinators is beneficial depends on the focal plant species in question (e.g. Kremen *et al.*, 2002; Gómez *et al.*, 2007; Davila *et al.*, 2012). Meta-analysis indicates that specialization is generally risky; plants visited by many pollinators (> 5 species) are less pollen limited than those visited by few (1–5) species (Knight *et al.*, 2005). The effects of pollinator diversity on plant reproduction may be obscured because visitation rates are often not an adequate reflection of pollination rates because pollinators vary in their effectiveness (e.g. King *et al.*, 2013), which could underlie why visitation rates do not often correspond with the level of pollen limitation (Muir & Vamosi, 2015). Previous attempts to tease actual pollination apart from observations of visits have revealed large differences in pollen transport networks vs visitation networks (Ballantyne *et al.*, 2015), yet these studies have had to resort to painstaking visual pollen identification procedures that require rare alpha taxonomic expertise. Although there are automated visual approaches, they are also expensive, time consuming, and need just as extensive a reference database as does DNA barcoding (Marcos *et al.*, 2015). Abundance and composition of heterospecific pollen transfer (HPT) has been determined to affect seed production and may underlie why oligolectic (specialist) pollinators heighten plant fitness more so than generalists (Arceo-Gómez & Ashman, 2011; Brosi & Briggs, 2013). If stigmas were collected after single visits from a variety of pollinators, it would be possible to gain estimates of each pollinator's mean quality of pollen deposition (in terms of the frequency of pure conspecific pollen loads identified through DNA barcoding).

While specialists likely contribute some level of pollination services in any given community, the labile nature of specialization complicates our interpretation of what elements of diversity we should strive to conserve. The ecosystem service of pollination is a community-level process, and therefore maintaining it may be difficult to reconcile with the current system of prioritizing species for conservation. The designation of species-at-risk is based on species attributes (e.g. range and population size) and may therefore fail to capture the functional role of a species (i.e. such habitat specialists are often diet generalists; Litsios *et al.*, 2014). By necessity, specialization is often considered a relatively invariant species-property that is consistent across locations (Devictor *et al.*,

Box 1 Examples of how barcodes can be used to examine plant–pollinator interactions

Pictures show (a) pollen of *Dipsacus asperoides* on the body of *Bombus festivus*, and (b) nectar extraction from *Delphinium tenii*. Plant species visited by a single pollinator can be determined with DNA metabarcoding, but reconstructing the list of pollinator species that visited a given plant from DNA barcode fragments in nectar is still in development. Obtaining these measurements for all floral visitors collected at a site, individual and community-level metrics of specialization can be constructed. Pairing these metrics with seed production values offers ways to determine what pollinator species, or combinations of species, play the greatest functional roles in communities. DNA barcoding permits these metrics to be evaluated at unprecedented spatial and temporal scales, especially if barcodes (even that of only cytochrome oxidase 1 (CO1)) are added to that of backbone phylogenies. Using historical ecology approaches will allow us to estimate whether the most critical pollinators in any given system are declining.



2010). Models that have incorporated spatial heterogeneity find that specialists should evolve when their habitat or mutualists become common (Whitlock, 1996; Débarre & Gandon, 2010). There have been few empirical tests of these models using host-plant use because they require community-level sampling over vast areas (Bridle *et al.*, 2013; Vamosi *et al.*, 2014a; Adderley & Vamosi,

Table 1 Summary of questions where DNA barcoding should prove useful in broadening the scope and precision of studies focused on understanding the relationship between diversity and pollination ecosystem services

Specialization question	Advantages of approach with DNA barcoding	Demonstrated potential
How does the level of specialization change when measured through pollen on pollinators vs visually observed visitors?	Examine whether pollen of plants visited is actually transported on insects by DNA barcoding pollen found on insects. Advantage: identifying pollen through microscopy is a labor-intensive process requiring extensive expertise; identifying to species-level is often not possible and has accuracy < 78% (Mander *et al.*, 2014) as opposed to ≤ 95% with pollen DNA barcoding.	Sickel *et al.* (2015); Richardson *et al.* (2015a); Galimberti *et al.* (2014)
How does pollinator sharing (i.e. specialization) change within a species' range (or as a function of pollinator species richness or disturbance)?	Standard DNA barcoding of plant community with multiplexed DNA barcoding of pollen sampled from pollinators and/or stigmas. Advantage: thus far, macroecological studies that follow species often reduce sampling to a very small number of communities, species, or individuals (e.g. Tur *et al.*, 2013; with 27 species, *c.* three individuals each, in two communities or 162 sampling units, whereas a single multiplexed run of pollen samples can contain double this number).	Kress *et al.* (2009); Pei *et al.* (2011); Bezeng *et al.* (2015); Clare (2014); Toju *et al.* (2014)
Is the rate of turnover of specialists higher than generalists in disturbed areas; is it elevated compared to historical levels?	Barcoding pollen on plant and pollinator specimens in collections and determine which interactions have been lost through time. Advantage: as mentioned earlier, DNA pollen barcoding removes the prohibitive time barrier of identifying the pollen through microscopy.	Shokralla *et al.* (2011)

2015). By assessing the pollen on pollinators through DNA barcoding across a species' range, we can determine to what degree this heterogeneity in ecological options throughout the geographic range of a species determines our measurement of pollinator specialization (Table 1). By accurately measuring the level of context-dependent ecological specialization in plant–pollinator interactions over longer timescales, we can examine how constancy over time has affected the evolution of pollinator specialization and determine the effects that losing specialists will have on pollination services.

Temporal variation in specialization and the stability of ecosystem services

While some heterogeneity in specialization occurs naturally, intensive human impacts result in a decrease in the degree of specialization in communities over time (Weiner *et al.*, 2014). Habitat fragmentation has been repeatedly observed to reduce pollination services and plant reproductive success (Knight *et al.*, 2005). Evidence suggests that specialists often decline with disturbance more readily than generalists (Packer *et al.*, 2005; Zayed *et al.*, 2005; Aguilar *et al.*, 2006; Weiner *et al.*, 2014). High nestedness of networks (the degree to which specialists employ subsets of the suite of species used by generalists) has been used as an indicator of greater robustness of networks (Burkle *et al.*, 2013). Empirical observations indicate that nestedness increases when specialists are present in a community, suggesting the loss of specialists does reduce the stability of ecosystems (at least, once the community is reduced to complementary generalists; Burkle *et al.*, 2013). With the goal of forecasting the effects of biodiversity loss, metabarcoding techniques, combined with the collection of georeferenced voucher specimens (Marques *et al.*, 2013), provide a means for extensive monitoring to reveal geographical hotspots of declines in bee populations should pollinator communities be assessed on a regular basis (Tang *et al.*, 2015). Considering the worrisome rate of range contraction of many bee species (Kerr *et al.*, 2015), these methods can be combined with extinction rates estimated through phylogenetic methods to help address whether the bee clades that are declining currently are the same as those that have historically suffered declines (Hardy & Otto, 2014) as well as what traits (such as levels of generalization in diet of pollen and nectar sources) allow for resilience of bee species (Groom *et al.*, 2014).

To what degree is the extirpation of specialist species part of a natural pattern of species turnover? We consider how DNA barcodes can detect historical rates of loss of specialists in two different ways: (1) using a historical ecological approach to detect specialization from museum and herbarium records over decades to centuries; and (2) using a phylogenetic approach to detect background extinction rates over evolutionary timescales. DNA barcoding of pollen on collected specimens allows for, in addition to the large-scale spatial analyses already described, large-scale temporal analyses. By metabarcoding the pollen on herbarium (in the case of floral stigmas) and museum (in the case of pollinators) specimens, it will be possible to compare historical levels of specialization to that of the present day and determine the number

and identity of species lost over time with increasing amounts of anthropogenic disturbance (Scheper *et al.*, 2014). This approach appears feasible as researchers have been successful at detecting DNA barcodes from museum specimens (Shokralla *et al.*, 2011), and DNA in pollen, in particular, appears amenable to deep-time synthesis (Suyama *et al.*, 1996; Parducci *et al.*, 2005).

Because commonly used plant barcode markers are phylogenetically informative, researchers have established that barcode sequences are useful in plant-community phylogenetics (Kress *et al.*, 2009; Pei *et al.*, 2011; Bezeng *et al.*, 2015). In such work, two to three markers are typically sequenced, including a gene that is broadly recognized for its utility for plant phylogenetics, *rbcLa* (CBOL Plant Working Group, 2009; Kress *et al.*, 2009; Pei *et al.*, 2011, 2015; Kuzmina *et al.*, 2012; Bezeng *et al.*, 2015). Thus, plant phylogenies reconstructed from barcodes are generally found to be accurate, i.e. when compared against phylogenies based upon more evidence (Kress *et al.*, 2009; Pei *et al.*, 2011, 2015). By contrast, single-marker (CO1) barcoding is generally performed for animals due to its effectiveness for recognizing the majority of species as revealed through traditional morphological or integrative methods (e.g. Buide *et al.*, 1998; Hebert *et al.*, 2003a,b; Smith *et al.*, 2006, 2008; Sheffield *et al.*, 2009; Ratnasingham & Hebert, 2013; Ondrejicka *et al.*, 2014; Schmidt *et al.*, 2015). Given the high rates of molecular evolution, biased substitution patterns, and matrilineal mode of inheritance of the mitochondrial genome (Lin & Danforth, 2004), barcode-based phylogenies for animals are generally not expected to be as robust as nuclear-gene phylogenies or the multi-gene phylogenies derived during plant barcoding. However, animal barcode data can increase the taxon density in a phylogenetic study when 'backbone' phylogenies constructed using multi-gene data or phylogenomics approaches constrain the deeper topology (e.g. genus or family-level relationships; see Trunz *et al.*, 2016), in combination with barcode data, which are available for more species (Wilson, 2011; Boyle & Adamowicz, 2015). By incorporating barcode markers, phylogenetic trees have better phylogenetic resolution, which can yield more precise conclusions regarding community structuring and processes (Kress *et al.*, 2009; Pei *et al.*, 2011; Davies *et al.*, 2012).

Application of phylogenetic comparative methods has indicated that specialization is labile; evolutionary transitions from specialization to generalization are common (Abrahamczyk *et al.*, 2014; Vamosi *et al.*, 2014b). However, extinction rate estimates from these studies do suggest that specialized branches on the tree of life are ephemeral relative to those of generalists (e.g. Colles *et al.*, 2009). Niche specialists are expected to have much smaller geographical ranges (Williams *et al.*, 2009) and be prone to greater extinction rates due to lower global abundance (Packer *et al.*, 2005), yet there are few empirical tests in pollinator clades (Hardy & Otto, 2014). Further study of plant and pollinator phylogenies will allow us to estimate the degree of background extinction of specialist lineages vs that of generalists as well as the propensity of specialists to undergo transitions to generalization under certain ecological conditions. With resolved phylogenetic trees we should be able to examine whether lineages that have historically experienced high levels of extinction are the same lineages that are now experiencing population declines from anthropogenic disturbance. Relating these findings further to pollinator effectiveness in pollen delivery should result in a shortlist of efficient pollinators that were historically robust but are now at risk, and which might, therefore, increase the risk for the plants they visit. Such approaches should form part of any decision-making, concentrating conservation efforts where they are most needed.

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