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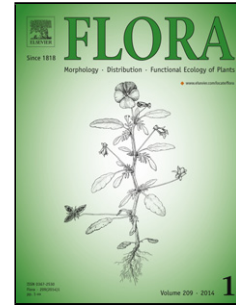
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Diversity of Diptera families that pollinate *Ceropegia* (Apocynaceae) trap flowers: an update in light of new data and phylogenetic analyses

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Highlights

- In *Ceropegia*, there are phylogenetic differences in the use of Diptera families
- Biogeographic patterns in pollinator exploitation are more limited
- Most taxa are relatively specialised at least to the level of Diptera family

ABSTRACT

Pollination by flies (Diptera) has been important to the diversification and ecology of the flowering plants, but is poorly understood in contrast to pollination by other groups such as bees, butterflies and birds. Within the Apocynaceae the genera *Ceropegia* and *Riocreuxia* temporarily trap flies, releasing them after a fixed, species-specific period of time, during which pollination and/or pollen removal occurs. This "trap flower" pollination system shows convergent evolution with unrelated species in other families and fascinated Stefan Vogel for much of his career, leading to ground-breaking work on floral function in *Ceropegia* (Apocynaceae). In this new study we extend the work of the latest broad analysis published by some of the authors (Ollerton et al., 2009 – Annals of Botany). This incorporates previously unpublished data from India and Africa, as well as recently published information, on the diversity of pollinators exploited by *Ceropegia*. The analyses are based on a more accurate phylogenetic understanding of the relationships between the major groups, and significantly widens the biogeographic scope of our understanding of fly pollination within *Ceropegia*. Information about the pollinators of 69 taxa (species, subspecies and natural varieties) of *Ceropegia* is now available. Twenty five families of Diptera are known to visit the flowers of *Ceropegia*, of which sixteen are confirmed as pollinators. Most taxa are pollinated by species from a single family. Overall, there were no major biogeographic differences in the types of Diptera that were used in

particular regions, though some subtle differences were apparent. Likewise there were no differences between the two major clades of *Ceropegia*, but clear differences when comparing the range of Diptera exploited by *Ceropegia* with that of the stapeliads. This clade, one of the largest in the Asclepiadoideae, is a fascinating example of a species radiation driven by an apparently relatively uniform set of pollinators.

Keywords: Apocynaceae, Asclepiadoideae, *Ceropegia*, Diptera, flower evolution, pollination, specialisation, Ceropegieae-Stapeliinae

1. Introduction

Current understanding of the global biodiversity of plant-pollinator interactions has tended to be biased towards angiosperms that are pollinated by relatively large, often charismatic insects, particularly bees and larger Lepidoptera, and vertebrates such as bats and birds. However a significant fraction of the flowering plants is pollinated by apparently unremarkable, easily over-looked insects including Diptera (Kearns, 2001; Larson et al., 2001; Ollerton et al., 2009, Ollerton in review). Studying such interactions can provide a fuller insight into the biodiversity of plant-pollinator relationships, and therefore the ecology and diversification of the angiosperms. The tribe Ceropegieae of the Apocynaceae is remarkable for its high proportion of fly pollinated species, particularly in the large genus *Ceropegia* and within the closely related stapeliads (Vogel, 1961; Meve & Liede, 1994; Ollerton & Liede, 1997). However the full diversity of fly families and genera exploited by *Ceropegia*, and the exact nature of the relationship between flowers and pollinators,

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has only recently been appreciated (Masinde, 2004; Ollerton et al., 2009; Coombs et al., 2011; Heiduk et al., 2010, 2015, 2016, 2017).

In addition to the studies of pollination in *Ceropegia* there has been a growing body of work aimed at understanding the complex evolutionary history of the genus in relation to related genera of Ceropegieae (Meve & Liede-Schumann, 2007; Bruyns et al., 2015; Meve et al., 2016). All studies agree that the long-tubed trap flowers of *Ceropegia* (Stapeliinae) are paralleled in *Riocreuxia* (Anisotominae), even though the two genera differ in coronal structures. Furthermore, open flowers of the *Brachystelma* type have evolved several times from *Ceropegia*-type flowers and the stem-succulent, open-flowered Stapeliads are also retrieved inside long-tubed *Ceropegia*. This provides an important phylogenetic framework for interpreting patterns of pollinator use within this clade.

What is particularly remarkable about this clade of plants is that they have diversified without major shifts between pollinator types, such as switches between bee, bird, bat and butterfly pollination, as has been observed in other taxa, e.g. Johnson et al., 1998; Kay et al. 2005; Muchhala 2006; Wilson et al., 2006; Castellanos et al. 2004; Smith et al. 2008. We examine this further in the Discussion.

The purpose of this paper is to extend the recent work on this group by synthesising additional records of pollinators of *Ceropegia* which were not available for the analyses by Ollerton et al. (2009), including data from the Indian subcontinent, one of six centres of particular diversity for *Ceropegia* that has so far been under-sampled (see Table 1 in Ollerton et al., 2009). In addition we use new phylogenetic analyses of *Ceropegia* (Fig. 4) to test the robustness of the conclusions drawn previously.

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The present study addresses the following questions: (1) What is our current understanding of the diversity of pollinators of *Ceropegia* and how has this understanding changed over time? (2) How does the diversity and specialisation of pollinators of *Ceropegia* compare across centres of diversity for the genus? (3) Does the improved phylogenetic understanding of the major groups of *Ceropegia* and allied taxa, and the additional data on pollinators, change our interpretation of the evolution of plant-pollinator interactions within this group of plants?

Answering these questions will give us a much fuller understanding of the diversity, variability and specificity of plant-pollinator interactions in *Ceropegia*, and of the ecology and evolution of fly pollinated plants more broadly.

2. Material and methods

2.1 Ceropegia *and its pollinators*

Our study focuses on *Ceropegia* L. (Apocynaceae: Asclepiadoideae, Ceropegieae), a large, paraphyletic (Meve & Liede-Schumann, 2007; Surveswaran et al., 2009; Bruyns et al. 2015) genus of more than 200 accepted species distributed from the Canary Islands in the west, across much of sub-Saharan Africa, Madagascar, the Arabian Peninsula, Southeast Asia (including the Indian subcontinent, Laos, Myanmar, Thailand and China), to the southwestern Pacific Region (including Indonesia, Philippines, Papua New Guinea and Northeast Australia). New species are frequently described (e.g. Bruyns, 2003; Malpure et al., 2006; Dold, 2006; Meve 2009; Thulin, 2009; Sujanapal, 2013; Punekar et al., 2013; Kidyoo, 2014; Kidyoo & Paliyavuth 2017) and the full diversity of the (currently paraphyletic) genus may exceed 250 species. Regardless of taxonomy, the Ceropegieae is one of the largest monophyletic clades within the Asclepiadoideae.

As with all Asclepiadoideae genera, flowers of *Ceropegia* present their pollen as coherent masses (pollinia) that mechanically clip as pollinaria onto the body of flower visitors, making it relatively straightforward to distinguish between true pollinators and non-pollinating flower visitors.

The records of pollinators used in this paper come from three sources: (1) published studies from the literature, including recent work not included in Ollerton et al. (2009); (2) dissection of flowers in the spirit collection of the Herbarium at the Royal Botanic Gardens, Kew, most of which were included in Ollerton et al. (2009), but including some additional records; (3) field work since 2009 by some of the authors which has resulted in new, unpublished records from India (SP & KG), Ethiopia (MT), the Canary Islands (MT & JO), western Africa (Porembski) and southern and eastern Africa (AH et al.) A full list of all records, with sources and accession numbers (as appropriate) is provided in Appendix 1.

All insects have been identified to at least family level by taxonomic specialists (KG, AW, and individuals cited in acknowledgements of Ollerton et al., 2009, and the publications of Heiduk and colleagues).

2.2 Data analysis

All pollinators so far identified for *Ceropegia* (this study included) have proven to be true flies (Diptera). The taxonomy of small sub-tropical and tropical Diptera is relatively poorly known so we have limited our analyses to the level of family for this particular study, to give a robust comparison with previous research. Future work will focus on the Diptera genera and species as identifications become available.

The phylogenetic analysis presented in section 2.3 shows that *Ceropegia* plus *Brachystelma* plus the stapeliads form a grade within which two major groups of *Ceropegia* can be identified. Although not technically the correct terminology, for simplicity we refer to these *Ceropegia* groups as Clade I and Clade II. To test for a clade effect (differences between Clade I versus Clade II) and region effect (see supplementary material) in presence and absence of Diptera families as pollinators of the different *Ceropegia* species, we performed a PERMANOVA analysis (fixed factors: clade, region; 10,000 permutations; only species with both region and clade information available were included) based on pairwise Sørensen similarities, using Primer 7.0.11 & PERMANOVA+1 add on. PERMANOVA was also used to test for differences in pollinator pattern among the five major centres of diversity of *Ceropegia* (see below; analysis was performed independent of clade membership of the species), and between the two major clades of *Ceropegia* and the stapeliads (factor: clade/plant group; 10,000 permutations; using all taxa of these groups with pollinators available), again based on Sørensen similarities. Differences in specialization between clades and regions (i.e. average number of families used as pollinators) were tested using Kruskal Wallis tests, and comparisons of similarities in, for example, use of pollinator families between regions and clades were made using Spearman Rank Correlations, carried out in SPSS 22. Visualisation of the interactions between *Ceropegia* spp. and fly families was conducted using the "bipartite" package in R (Dormann et al. 2008, R Core Team, 2014).

2.3 Phylogenetic methods

The dataset was assembled to comprise as many *Ceropegia* / *Brachystelma* species as possible, using a combination of the data of Meve & Liede-Schumann

(2007), Surveswaran et al. (2009) and Bruyns et al. (2015), augmented by some well documented GenBank sequences of other sources and 194 partial sequences newly created for the present study. For stem-succulent stapeliads, the dataset was restricted to twenty representative taxa, comprising, as far as possible, the species for which pollinator data are available (Appendix 1). Because the available datasets (Meve & Liede, 2002, Bruyns et al., 2005, 2014) often contain identical taxa, but differ slightly in markers analysed, sequences of two accessions of the same taxon were combined for 11 species, after comparison of partial sequences available for both accessions were found to be identical, or almost so (99%). The outgroup was assembled from Meve et al. (2017), comprising representatives of all genera of Heterosmminae, Anisotominae and Leptadeniinae. Appendix 2 details species, vouchers and GenBank accession numbers.

Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Six plastid markers (*trn*H-*psb*A, *trn*S-G, *trn*T-L and *trn*L-F intergenic spacers, *trn*L and *rps*16 intron) as well as the Internal Transcribed Spacer region (ITS), including 5.8S of ribosomal DNA (rDNA) were amplified following the procedures detailed in Meve et al. (2017).

For all new partial sequences, forward and reverse sequences were aligned with CodonCode Aligner, v.3.0.3 (CodonCode Corp., Dedham, Massachusetts, USA), and the consensus was exported in fasta format. The seven partial matrices were assembled inside Mesquite v. 3.0 and aligned with the OPAL package (Wheeler & Kececioglu, 2007); the resulting alignments were corrected manually. For the *trn*H-*psb*A spacer, outgroup sequences were removed, because they could not be aligned with confidence. No well-supported incongruence was found between the partial matrices, in accordance with previous studies in Ceropegieae (Bruyns et al., 2014,

2015; Meve & Liede-Schumann, 2007; Meve et al., 2017), therefore, the matrix was exported using the "Fused Matrix Export (Phylip / RAxML)" option of Mesquite. The Maximum Likelihood tree was calculated using RAxML v. 8.2.9 (Stamatakis, 2014) as implemented on the CIPRES platform (Miller et al., 2010) with automatically stopped bootstrapping and implementing a mixed partition model to allow different evolutionary rates for every partition.

2.4 Phylogenetic signal in pollinators

To test for a phylogenetic signal in use of pollinators (based on the presence/absence of pollinating fly families in the different plant taxa for which both pollinator and genetic data were available), we used Phylogenetic Principal Components Analysis (pPCA) (Jombart et al. 2010) and Pagel's λ (see Prieto-Benítez et al., 2016). pPCA creates two principal components (PCs) that summarize the phylogenetic resemblance in the phylogeny (see previous section) owed to pollinators. The first PC has the largest eigenvalues (large variance and strong positive autocorrelation) and the last PC has the lowest eigenvalues (high variance and strong negative autocorrelation). In this way, the first PC denotes pollinators that are more similar in related species and thus are phylogenetically constrained; the last PC denotes pollinators that create dissimilarities between closely related species. For this analysis, we used the measure of phylogenetic proximity underlying the test of Abouheif (1999) because of its abilities in detecting phylogenetic signal (Pavoine et al., 2008). As pPCA does not explicitly test for the presence of a phylogenetic signal, we used Pagel's λ to test for a phylogenetic signal in the PCs (see Freckleton et al., 2002; Prieto-Benítez et al., 2016).

As we detected a strong effect in the PCs of the clade formed by non-*Ceropegia* stapeliad species (*Orbea*, *Stapelia*, *Desmidorchis* and *Apteranthes*) and *Ceropegia bulbosa*, and a strong phylogenetic signal in PC1 (see Results), we performed a second pPCA that excluded these species in order to test whether there is a phylogenetic signal in pollinators also outside this clade. Pagel's λ detects phylogenetic dependence of an observed trait (values of λ approximately 1 indicates phylogenetic signal, λ approximately 0 indicates phylogenetic independence) (Freckleton et al., 2002). All analyses were implemented in R (R Core Team, 2014); we performed the pPCAs using the package "adephylo" (Jombart & Dray, 2008), and Pagel's λ using the function fitContinous in the package GEIGER (Harmon et al., 2008).

3. Results

The records presented in Appendix 1 indicate that we currently have information on the pollinators (as distinct from simply flower visitors) of 69 taxa (species, subspecies and natural varieties) of *Ceropegia*, almost twice as many as were available for the previous analysis by Ollerton et al. (2009). The following analyses focus only on those *Ceropegia* taxa for which pollinator identity has been confirmed (i.e. the Diptera that carried pollinaria).

3.1 Diversity of pollinators of Ceropegia

Sixteen families of Diptera are known to act as pollinators of *Ceropegia* taxa, whilst a further nine are visitors but unproven pollinators (Table 1). As Fig. 1 demonstrates, our understanding of the diversity of pollinators for these plants has increased almost linearly since the early 2000s, following a long period where a

limited diversity of pollinators had been identified. The family accumulation curve in Fig. 1 shows no sign of levelling off, indicating that the true family-level diversity of *Ceropegia* pollinators may be much higher. Indeed we have records of a further nine Diptera families which are known to visit flowers of *Ceropegia* but have not yet been confirmed as pollinators (Table 1). Further work may prove some of these to be insect predators or plant parasites, but others are almost certain to be pollinators (indeed one of them - Tephritidae - is known to pollinate at least one stapeliad species – see Appendix 1).

3.2 Pollinator specialisation at a Diptera family level in Ceropegia

There is a very right-skewed pattern to the distribution of the number of pollinating Diptera families, with more than three quarters of the *Ceropegia* taxa for which we have identified the fly family being pollinated by a single family of flies (Figs. 2 and 3). Some of these records are based on limited collections of flowers with pollinators and thus it is possible that a proportion of these taxa are more generalised in their interactions with pollinators than we currently know. But nonetheless a striking feature of *Ceropegia* is the high degree of Diptera family-level specialisation. There are some exceptions to this, however, as 17 taxa are pollinated by up to four Diptera families, and two species are pollinated by seven. As Fig. 3 shows the latter species are the very large flowered *C. ampliata* from southern Africa (Coombs et al., 2011) and *C. aristolochioides* subsp. *deflersiana* from the Arabian Peninsula, part of a species complex (Meve et al., 2001) that was previously identified as being much more generalised in its interactions with pollinators than other *Ceropegia* taxa (Ollerton et al., 2009). Both of these taxa have been extensively sampled compared with most other *Ceropegia* (Coombs et al., 2011; Appendix 1),

which may also be a factor (see also comments below on sampling effort). The focus on Diptera family, however, masks greater specialisation at the genus and species level which we will not immediately address in these analyses but which has been dealt with preliminarily by Ollerton et al. (2009; see also Heiduk et al. 2017).

3.3 Biogeographic patterns and the effect of main clades in the exploitation of pollinators in Ceropegia

Overall, there were no differences in the use of pollinator families among different regions (PERMANOVA: pseudo-$F_{11,46}$ = 0.93, P = 0.65), and between Clade I and Clade II (PERMANOVA: pseudo-$F_{1,46}$ = 0.64, P = 0.68). There was also no significant effect of the interaction between regions and clades (PERMANOVA: pseudo-$F_{2,46}$ = 0.84, P = 0.61). Most data are available for five of the major centres of diversity of *Ceropegia* - the Indian subcontinent, the Arabian Peninsula, East Africa, southern Africa, and West Africa (Fig. 4; Appendix 1). When including only species which occur in one of these centres in the analysis, and neglecting the clade membership, there also was no difference in the use of pollinators within the different regions (PERMANOVA: pseudo-$F_{4,51}$ = 1.08, P = 0.36). In all these regions the families Milichiidae and Chloropidae are important pollinators, accounting for between 10% and almost 30% of the pollinator records.

The biogeographic PERMANOVA analyses show no difference because variation within regions is very high, masking some of the more subtle differences of the frequency of particular families in certain parts of the world; for example the Indian subcontinent taxa frequently exploit the families Drosophilidae, Mycetophilidae and Cecidomyiidae, which are rare or unknown as pollinators in other regions (Fig. 4, Table 2). Likewise Phoridae is an important family of pollinators in

West Africa but is less often exploited in other regions (Fig. 4) though West Africa is less well represented in our analyses than the other regions and this pattern could change with additional sampling.

The Indian subcontinent and southern Africa share the most diverse pollinator family profiles, with eleven families each; however, these families are rather different and the profiles for the two regions are not correlated (Table 2). The addition of so much new data from the endemic taxa of the Indian subcontinent has added three new families (Anthomyiidae, Cecidomyiidae and Mycetophilidae) as pollinators of *Ceropegia*, though two of them (Cecidomyiidae and Mycetophilidae) had previously been recorded as flower visitors (Ollerton et al., 2009). Indeed, across all seven regions there is a strong positive correlation between the number of records of pollinators and the diversity of fly families used (Spearman rank correlation r = 0.93, n = 7, p = 0.003) which suggests that conclusions about the diversity of pollinators in a particular region are strongly influenced by sampling effort.

The specificity of the interactions between *Ceropegia* and its pollinators (at Diptera family level) was examined by comparing the average number of families used per *Ceropegia* taxon in each region for which there is sufficient data (Fig. 4). Overall there is no difference in the average number of fly families exploited as pollinators; mean (median) ± SD [range] number of families: Arabian Peninsula = 2.1 (1.0) ± 2.2 [1-7]; East Africa = 1.4 (1.0) ± 0.8 [1-3]; Indian subcontinent = 1.5 (1.0) ± 0.8 [1-4]; southern Africa = 1.6 (1.0) ± 1.6 [1-7]; West Africa = 1.8 (1.0) ± 1.2 [1-4] (Kruskal-Wallis test χ^2 = 1.17, d.f. = 4, p = 0.88).

3.4 Pollinators of the major clades of **Ceropegia** *and related taxa*

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In accordance with the latest phylogenetic analysis of *Ceropegia* and its relatives our tree shows that the group is broadly divided into two major clades that we refer to as Clade I and Clade II (Fig. 5). The genus *Brachystelma* is scattered throughout the phylogeny and is polyphyletic, thereby causing multiple paraphyly of *Ceropegia* (Bruyns et al. 2015). The stem-succulent stapeliads, a diverse group of c. 400 species in 47 genera (sensu Endress et al. 2014), are nested within Clade II. Comparison of the use of fly families as pollinators by the two major clades of *Ceropegia* with that of the stapeliads s. str. (Fig. 5) shows that these three groups overall exploit different fly pollinators (PERMANOVA: pseudo-$F_{2,76}$ = 6.93, P < 0.001). Post-hoc analyses revealed that the two distantly related *Ceropegia* clades exploit the same spectrum of fly families (P = 0.24), particularly Milichiidae, Chloropidae and Ceratopogonidae (which are rare or unknown as pollinators in the stapeliads); in contrast these two clades are dissimilar in their use of pollinators compared to the stapeliads (P < 0.001 each). This is in spite of the fact that the stapeliads are more closely related to *Ceropegia* Clade I than the latter is to *Ceropegia* Clade II. However a caveat to this analysis is that the pollination ecology of the stapeliads has been poorly studied to date (Appendix 1) and future data may change these results.

There is no difference in the average number of fly families pollinating *Ceropegia* and stapeliad taxa; [mean (median) ± SD [range] number of families: Clade I = 1.5 (1.0) ± 1.3 [1-7]; Clade II = 1.5 (1.0) ± 1.2 [1-7]; stapeliads = 2.0 (1.0) ± 1.7 [1-7] (Kruskal-Wallis test χ^2 = 1.99, d.f. = 2, p = 0.37).

3.5 Phylogenetic signal in the use of pollinators by Ceropegia

Phylogenetic principal component analyses summarized the phylogenetic signal due to the 17 Diptera families that pollinate *Ceropegia* (Fig. 7A). In the first pPCA, Muscidae and Calliphoridae, which mostly pollinate the stapeliad clade formed by taxa of *Orbea*, *Stapelia*, *Desmidorchis*, *Apteranthes*, as well as *Ceropegia bulbosa*, had the highest positive loading on PC1 (Fig. 7B). Chloropidae had the highest negative loading on the last PC (PC16, Fig. 7B) of the analyses. The presence/absence of this fly family varied most among closely related taxa, such as between the two subspecies of *C. aristolochioides*, and between *C. attenuata* and *C. anantii*. In the second pPCA, which excluded the non-*Ceropegia* clade together with *C. bulbosa* from the analysis, Milichiidae and Ceratopogonidae had highest positive and negative loadings on PC1, respectively (Fig. 7C). Milichiidae occur as pollinators of several closely related species, such as the clade formed by *C. sandersonii*, *C. radicans*, *C. nilotica*, *C. denticulata* and *C. stenantha*, and the clade formed by *C. longifolia* and *C. dolichophylla*. Ceratopogonidae pollinate several species of other clades, such *C. linearis*, *C. barklyi* and *C. pachystelma*. As in the first pPCA, the last PC was again strongly influenced by Chloropidae (negative loading in PC12, Fig. 7C). As expected, the first PC had a significant phylogenetic signal in both pPCAs ($\lambda = 1.0$; P <0.002 each), but not the last PCs ($\lambda = 0.0$; P >0.05 each).

4. Discussion

4.1 Specialisation and generalisation in Ceropegia *pollination ecology*

The functional details and floral Bauplan of the trap-flower "Kesselfallen-Blüten" of *Ceropegia* have been studied for some time (Knuth, 1909; Vogel, 1961,

1993; Endress, 1996; Coombs et al., 2011) though the diversity of Diptera pollinators has only recently been fully appreciated (Masinde, 2004; Ollerton et al., 2009; Coombs et al., 2011). In addition the chemical make up of the scents produced by some species has recently started to be investigated, indicating complex odour mimicry of dead insects by at least some of these flowers (Heiduk et al., 2010, 2015, 2016, 2017). Current evidence suggests that the sets of floral and vegetative characters that define the genus *Ceropegia* have been lost multiple times, and that the open flowers of *Brachystelma* and the stapeliads, for example, are derived from the trap flowers of *Ceropegia* (Meve & Liede-Schumann, 2007; Bruyns et al., 2015). Therefore convergent evolution driven by pollinators has probably resulted in similar floral phenotypes (including colour and scent) in the different clades of *Ceropegia* (and in *Riocreuxia*) compared to *Brachystelma*/stapeliads. Whilst trap flowers are certainly ancestral in this clade additional sampling in poorly represented parts of the phylogeny would be required to understand whether this comprises a symplesiomorphy for the group. Along these lines, floral scent is highly variable among species, phylogenetically not constrained, and may explain pollinator specificity in the more specialised members of this group of plants (Heiduk et al., 2017). It is clear from the considerable phylogenetic and ecological diversity of Diptera families exploited as pollinators of this genus (Table 1) that the evolution of plant-pollinator relationships within the group has been hugely complex. All the more remarkable is the fact that all of this floral evolution has (as far as we currently understand) been driven by a single taxon (Diptera). In contrast, floral diversity in most other plant groups so far studied is linked to evolutionary shifts between phylogenetically distinct pollinators, such as various combinations of bees, hummingbirds, butterflies, moths, bats, flies, etc. (e.g. Johnson et al., 1998; Kay et al.

2005; Castellanos et al. 2004; Muchhala 2006; Wilson et al., 2006; Smith et al., 2008). This may in part be due to researcher bias, such that plant clades with highly divergent flowers visited by diverse pollinators are more attractive as study systems. In contrast groups of plants that are pollinated by a single (albeit diverse) taxon may be relatively neglected (though see Armbruster et al. 2009). However it also tells us that even an apparently phylogenetically constrained set of pollinators such as the Diptera can select for significant levels of floral novelty, and reproductive isolation, and drive an evolutionary radiation involving hundreds of species.

The additional data obtained since the publication of Ollerton et al. (2009), particularly for species of *Ceropegia* endemic to the Indian subcontinent, conforms to the general view of pollination systems of *Ceropegia* taxa from other parts of the range of the genus. In the terminology of Ollerton et al. (2007) these species are phenotypically and functionally specialised, with clear adaptations to being pollinated by a single functional group of pollinators, namely small Diptera. However, at a Diptera family level, species may be ecologically specialised (using only one family of flies) or more generalised, exploiting up to seven different families. These findings are largely in line with the analyses of Ollerton et al. (2009) who likewise found both highly specialised and more generalised interactions within the genus (see also Coombs et al., 2011, Heiduk et al., 2017). However, given our comments above about the radiation of this group of plants, it is clear that there is probably considerable within-family functional diversity of these flies, in relation to ecological traits such as egg laying sites. This question will be addressed in the future once all of the flies are identified to at least genus, and more is discovered about their ecology.

4.2 Diversity of Diptera pollinators

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The present analysis significantly extends the phylogenetic breadth of Diptera known to be pollinators of this large genus. Particularly noteworthy is the family Cecidomyiidae that Ollerton et al. (2009) observed to be frequently found in *Ceropegia* flowers but never to carry pollinaria, perhaps because Cecidomyiidae are usually herbivores of buds and flowers, plant gall-making parasites, or predators of small invertebrates. The discovery of Cecidomyiidae as pollinators of four species of Indian *Ceropegia* significantly adds to the range of plant families known to be pollinated by these gall midges, which includes species within Monimiaceae (Feil, 1992), Sterculiaceae (Young, 1985), Schisandraceae (Luo et al., 2010), Araceae (Barriault et al., 2009), Moraceae (Sakai et al., 2000) and probably Piperaceae (Ollerton, 1996).

There are a little over 180 families of Diptera worldwide, the adults of which (barring some exceptions) require a sugar meal for energy and reproduction, much of which is derived from floral nectar. Clearly, this is the driving force behind floral visitation to most groups of plants, which in turn leads to pollination, as few are obligate pollen feeders such as Syrphidae, Bombyliidae, some Ceratopogonidae and some Phoridae (Larson et al., 2001). Noteworthy is that their larger size excludes Syrphidae and Bombyliidae from pollination of trap flower Apocynaceae, whereas the small bodied Ceratopogonidae and Phoridae are known pollinators of this family.

Entrapment of the flies is clearly a physical attribute of *Ceropegia* flowers that we can visually measure (floral shape and orientation, trichomes that collapse, presence of pollinia), whereas odour attraction has largely been overlooked until recently (Heiduk et al., 2010, 2015, 2016; but see Vogel 1961) and clearly plays a major role in plant-pollinator interactions within the Apocynaceae. Besides prey mimicry of the type described by Heiduk et al. (2010, 2015, 2016) there is also the

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possibility that mate attraction might play some role, with the flies being attracted by odours emulating fly pheromones. Mimicry of breeding and egg laying sites was also suggested to occur in the genus (Vogel, 1961, 1993; Ollerton et al., 2009; Heiduk et al., 2017), but the mimicry strategy of most of the species remains unexplored.

The presence of nectar in at least some *Ceropegia* species (Coombs et al., 2011) indicates that either not all species are deceptive or that nectar-secreting species are partly deceptive as they advertise another reward through scent mimicry. The presence of pollinaria only on the mouthparts of flies strongly suggests that nectar is used to manipulate the behaviour of the flies in the trap flowers as the nectar-bearing "cups" of Ceropegieae are all positioned below the guide rails of the gynostegium, where fly mouthparts can be steered to the pollinaria. If this were not the case then we would expect pollinaria to be clipped to legs and body hairs due to random movements of the flies in the flowers. Thus the kleptoparasitism of species of Milichiidae (Heiduk et al., 2010, 2015, 2016) is an aspect of a more general feeding behaviour in these flies centred on liquids, as they do not possess biting mouthparts.

The fly pollinators so far identified for Apocynaceae cover the phylogenetic spectrum of Diptera, ranging from small Culicomorpha, through Platypezoidea, Psychodomorpha, Bibionomorpha and small acalyptrate Schizophora, to large calyptrate Schizophora in the Muscoidea and Oestroidea (Meve & Liede, 1994; Ollerton & Liede, 1996; Ollerton et al., 2009; this study). The majority of families encountered pollinating Apocynaceae belong to the Acalyptratae (Schizophora) and therein to the Milichiidae, Chloropidae and Drosophilidae. While Milichiidae and Chloropidae share close phylogenetic affinities (and may even be sister taxa - see Buck, 2006) Drosophilidae are loosely grouped with the Ephydridae. Such a broad span of taxa does not easily map onto the phylogenetic structure of Apocynaceae,

indicating that there has been no close co-evolution between these interacting clades. It further suggests that fly body size in comparison with flower size governs associations (see also Ollerton et al., 2009).

4.3 Phylogenetic patterns of pollinator use in Ceropegia

The phylogenetic analyses of pollinator use undertaken by Ollerton et al., (2009) can now be largely disregarded as it is clear that the underlying phylogeny used in that study was not an accurate reflection of the evolutionary history of this group. At the present time we can say that there are both similarities and differences in the range of flies used as pollinators by the two major clades of *Ceropegia* (Clade I and Clade II) and that both of these, in turn, are very different from that of the stapeliads s.str. (Fig. 5 and Fig. 6). Also, we can conclude that there is phylogenetic signal in the use of pollinators, being somewhat constrained within *Ceropegia* (which may correlate with fly size – see above), and also when comparing *Ceropegia* with the stapeliads (see also Heiduk et al., 2017). Future work should focus on the pollination ecology of the stapeliads s. str. which, despite being a well-studied group taxonomically and horticulturally, are rather neglected ecologically (but see Meve et al., 2004, Jürgens et al., 2006), and on the genus *Brachystelma*. The latter shows many convergent features of floral phenotype with the stapeliads s. str. but there are almost no published data on their pollination biology.

4.4 Biogeographic patterns of pollinator use in Ceropegia

Biogeographic patterns of pollinator use were previously assessed by Ollerton et al. (2009) who found that the region with the greatest diversity of pollinating families of Diptera was the Arabian Peninsula region. The current work, plus the

study by Coombs et al. (2011) allows a reassessment of this, and it is clear that both the Indian subcontinent and southern Africa surpass the Arabian Peninsula in this respect. However this is unlikely to be the last word on the subject as there is known to be a significant effect of sampling effort on plant-pollinator surveys, including large-scale biogeographic assessments (Ollerton & Cranmer, 2002; Herrera, 2005; Ollerton et al., 2003, 2009) and the present study hints that this will continue to be the case for some time until *Ceropegia* has been more thoroughly sampled (see also comments above regarding *C. ampliata* and *C. aristolochioides* subsp. *deflersiana*). Interestingly, the present work also shows that the taxa of different regions exploit overall the same pollinator groups, indicating that *Ceropegia* species make use of very widespread pollinator families. Advances in Dipterology in tropical and other high-diversity regions will also improve our ability to both identify taxa and understand their behaviour, and reveal how this might be manipulated by *Ceropegia*.

4.5 Ceropegia *pollination in comparison to other Ceropegieae*

The other subtribes of Ceropegieae interact with a wide range of different groups of pollinators, including Diptera; in Anisotominae, sister group of Stapeliinae, *Sisyranthus trichostomus* is beetle pollinated (Ollerton et al., 2003) and *Riocreuxia* spp. are fly pollinated (Heiduk et al., in prep.), whilst in the Leptadeniinae *Orthanthera albida* is moth pollinated (Nel, 1995), *Leptadenia reticulata* is mainly bee pollinated (Pant et al., 1982; Chaturvedi & Pant, 1986), *L. pyrotechnica* is mainly pollinated by flies and beetles (Ali, 1994), and *L. madagascariensis* is pollinated by a range of flies from the family Drosophilidae (Yassin et al., 2012). In all of these examples pollinaria were placed on the mouthparts of the insects concerned and, indeed, such placement of pollinaria may be one of the defining features of the

pollination biology of this clade, in comparison with (for example) clades such as *Asclepias* and its relatives that place pollinaria on a range of body parts, including legs and abdominal and thoracic hairs, instead of or in addition to mouthparts (Ollerton et al., 2003).

5. Conclusion

This study has significantly extended our current understanding of the diversity of pollinators of *Ceropegia* at the level of Diptera family, and has demonstrated how this has changed over the past 55 years. Future sampling will no doubt add to our knowledge of the range of families exploited by *Ceropegia* (and related taxa) and more detailed studies such as Coombs et al. (2011) and Heiduk et al. (2010, 2015, 2016, 2017) will provide important insights into the mechanisms by which pollinators are attracted and trapped.

It is clear that whilst there are phylogenetic differences in the use of Diptera families, biogeographic patterns in pollinator exploitation are more limited, and that most taxa (with some exceptions) are relatively specialised (at least to the level of Diptera family). Although there is much still to discover, this study has helped to build a clearer picture of the biodiversity of plant-pollinator interactions within this remarkable group of plants, a process that was aided considerably by the foundational work of Stefan Vogel.

Supplementary information

Appendix 1: Records of pollinators for *Ceropegia* and related stapeliads

Appendix 2: Details of species, vouchers and GenBank accession numbers used in the phylogenetic analysis.

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Figure legends:

Fig 1: Cumulative number of Diptera families confirmed as pollinators of *Ceropegia* over time, from initial studies in the 1960s. Note that Vogel's (1961) study was of plants in cultivation, outside the natural range of the genus.
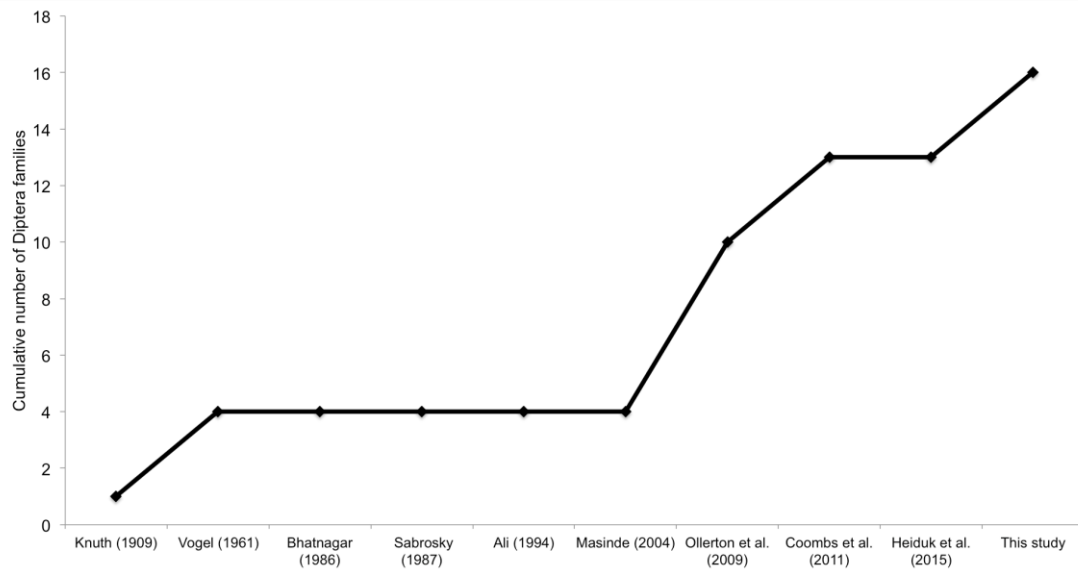
Fig 2: Frequency distribution of number of fly families acting as pollinators per *Ceropegia* taxon.
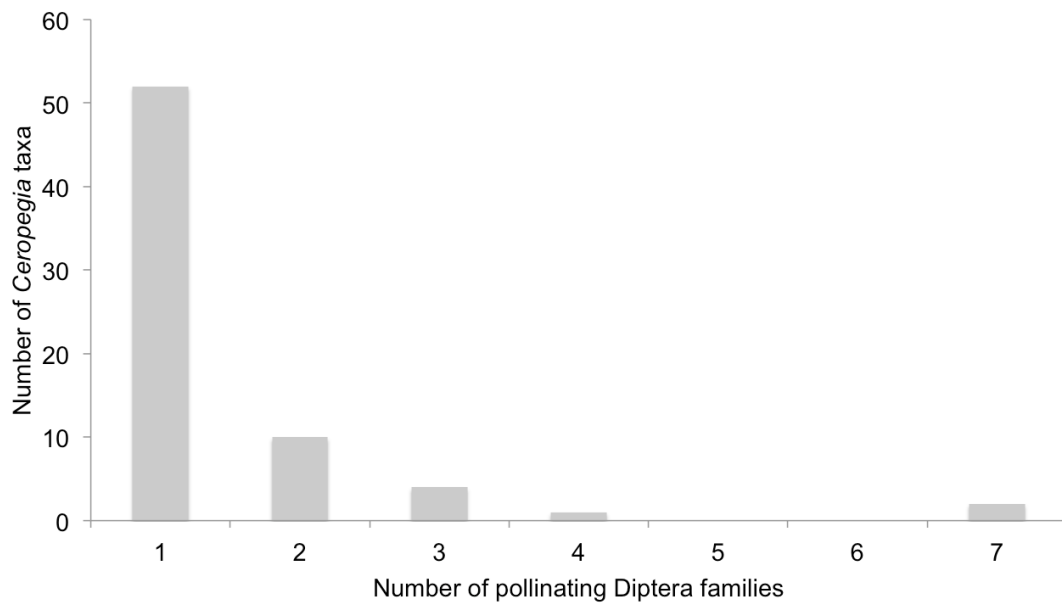
Fig. 3: Interactions between *Ceropegia* taxa and pollinating fly families represented as a bipartite graph. The box sizes are proportional to the level of specialisation of each *Ceropegia* taxon, and the number of *Ceropegia* taxa known to interact with a particular fly family, respectively. Plants are ranked from most generalist to most specialist; fly families are ranked from least exploited to most exploited.
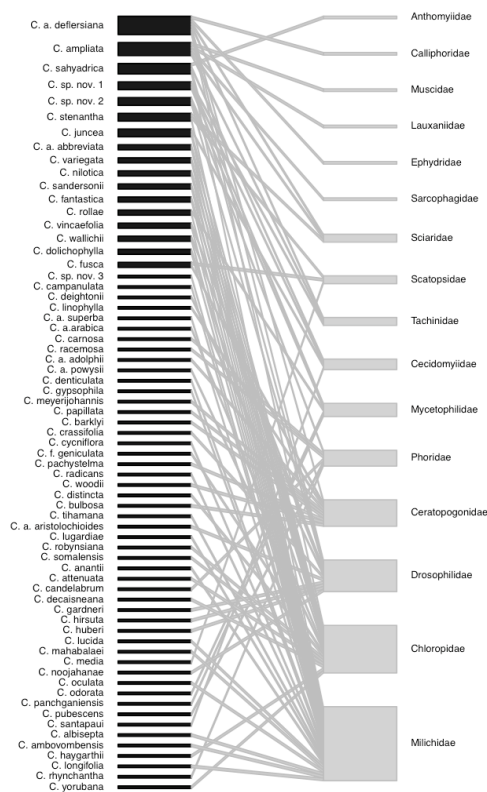
Fig 4: Comparison of the use of fly families as pollinators between five of the major biogeographic centres of distribution of *Ceropegia*. Data are plotted as the proportion of occurrences of each family in *Ceropegia* taxa from those regions.
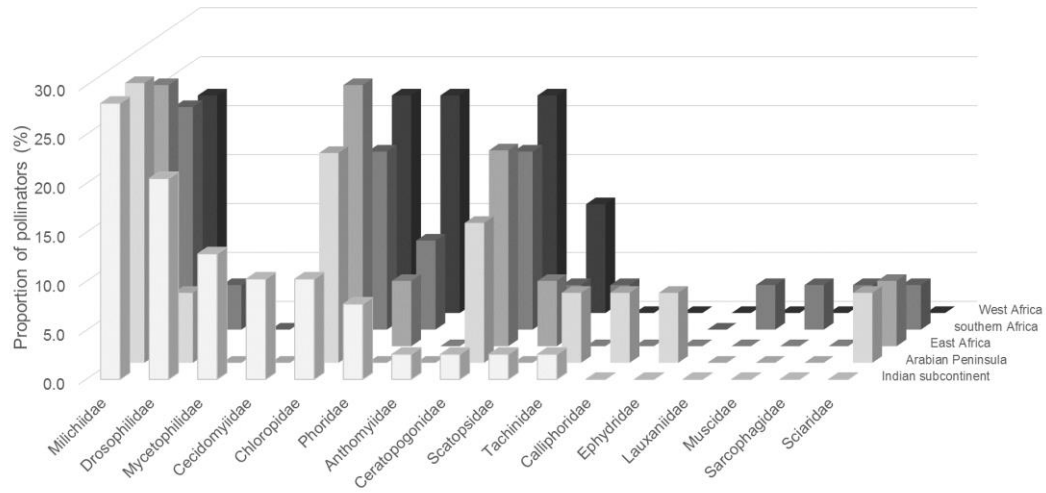
Fig 5: Maximum likelihood (ML) phylogenetic tree of Ceropegieae obtained with RAxML (Stamatakis 2014) based on combined trnT-F region, rps16 intron, trnH-psbA and trnS-G spacers, and ITS data. Bold lines indicate bootstrap support of 100 %, medium lines 90-99 % and slightly thickened lines 75-89 %. For some major clades, bootstrap values have been added directly to the branches. Taxa in bold indicate species for which pollinator data are available. For clarity, outgroups and the two large *Brachystelma* clades, for which no pollinator data are available, have been summarized as triangles.
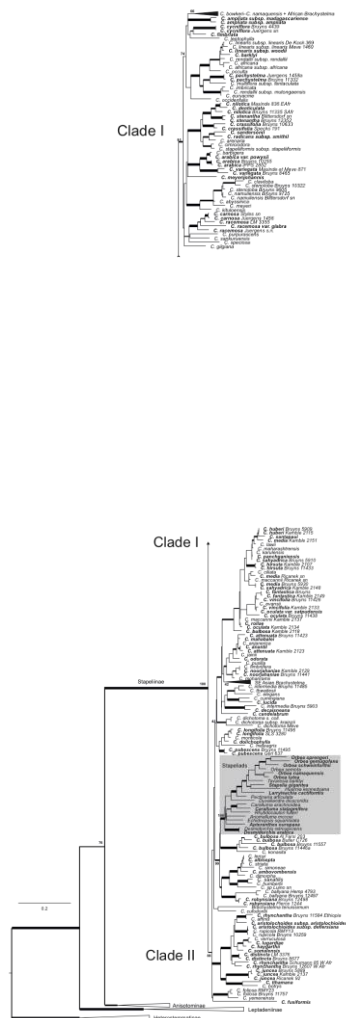
Fig 6: Comparison of the use of fly families as pollinators by the two major clades of *Ceropegia* (Clade I and Clade II), with that of the stapeliads s.str. Data are plotted as the proportion of occurrences of each fly family in taxa belonging to those clades.

Fig. 7. (A) Phylogeny of the species studied and results of the first (1st) and second (2nd) pPCAs. Positive and negative scores on PC1 (1st and 2nd), PC16 (1st) and PC12 (2nd) are indicated by black and white circles, respectively. Symbol size is proportional to absolute values. (B) Loading of the pollinators for the PC1 and PC16 in the first pPCA. (C) Loading of the pollinators for the PC1 and PC12 in the second pPCA.
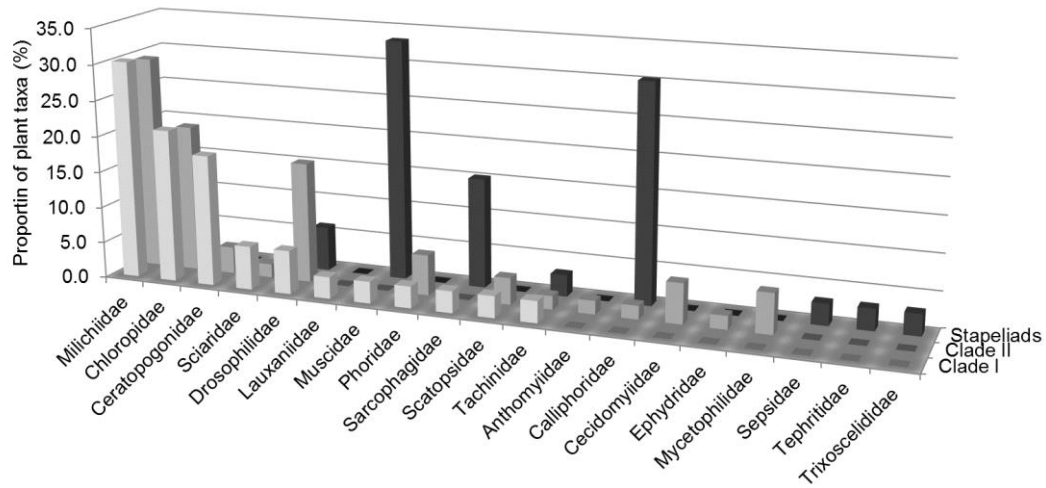
Table 1: Synopsis of the higher-level phylogenetic positions of Diptera families known to be flower visitors to *Ceropegia*. Confirmed pollinators are shown in **bold**. Phylogenetic information is from the Tree of Life project (www.tolweb.org).

Higher clade	Families	Common name
Schizophora	Agromyzidae	Leaf-miner flies
Schizophora	**Anthomyiidae**	Anthomyiid flies
Schizophora	Asteiidae	Asteid flies
Schizophora	**Calliphoridae**	Blow flies
Schizophora	Carnidae	Carnid flies
Schizophora	**Chloropidae**	Frit flies
Schizophora	**Drosophilidae**	Fruit flies
Schizophora	**Ephydridae**	Shore flies
Schizophora	**Lauxaniidae**	Lauxanid flies
Schizophora	**Milichiidae**	Milichid flies
Schizophora	**Muscidae**	House flies
Schizophora	**Sarcophagidae**	Flesh flies
Schizophora	**Tachinidae**	Tachinid flies
Schizophora	Tephritidae	Fruit flies
Bibionomorpha	**Cecidomyiidae**	Gall midges
Bibionomorpha	Lygistorrhinidae	Fungus gnats
Bibionomorpha	**Mycetophilidae**	Fungus gnats
Bibionomorpha	**Sciaridae**	Dark-winged fungus gnats
Culicomorpha	**Ceratopogonidae**	Blood-sucking midges
Culicomorpha	Chironomidae	Non-biting midges
Empidoidea	Empididae	Dagger flies
Empidoidea	Hybotidae	Dance flies
Platypezoidea	**Phoridae**	Scuttle flies
Psychodomorpha	Psychodidae	Moth flies
Psychodomorpha	**Scatopsidae**	Scatopsid flies

Table 2: Spearman rank correlations of the proportional use of different fly families as pollinators between the major centres of diversity of *Ceropegia*. Statistically significant correlations at $p \leq 0.05$ are indicated in bold; note that two of the West Africa comparisons are marginally significant at $p \leq 0.10$. $N = 16$ in each case.

	Arabian Peninsula	East Africa	Southern Africa	West Africa
Indian subcontinent	r = 0.24, p = 0.37	**r = 0.50, p = 0.05**	r = 0.28, p = 0.29	r = 0.45, p = 0.08
Arabian Peninsula		**r = 0.63, p = 0.009**	**r = 0.49, p = 0.05**	r = 0.42, p = 0.10
East Africa			**r = 0.82, p < 0.001**	**r = 0.84, p < 0.001**
Southern Africa				**r = 0.80, p < 0.001**