Molecular Phylogenetics and Evolution 115 (2017) 95–105

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Playing with extremes: Origins and evolution of exaggerated female forelegs in South African *Rediviva* bees

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ARTICLE INFO

Keywords:
Molecular phylogenetics
Plant-pollinator interaction
Ecological adaptation
Greater cape floristic region
Trait evolution
Melittidae

ABSTRACT

Despite close ecological interactions between plants and their pollinators, only some highly specialised pollinators adapt to a specific host plant trait by evolving a bizarre morphology. Here we investigated the evolution of extremely elongated forelegs in females of the South African bee genus *Rediviva* (Hymenoptera: Melittidae), in which long forelegs are hypothesised to be an adaptation for collecting oils from the extended spurs of their *Diascia* host flowers. We first reconstructed the phylogeny of the genus *Rediviva* using seven genes and inferred an origin of *Rediviva* at around 29 MYA (95% HPD = 19.2–40.5), concurrent with the origin and radiation of the Succulent Karoo flora. The common ancestor of *Rediviva* was inferred to be a short-legged species that did not visit *Diascia*. Interestingly, all our analyses strongly supported at least two independent origins of long legs within *Rediviva*. Leg length was not correlated with any variable we tested (ecological specialisation, *Diascia* visitation, geographic distribution, pilosity type) but seems to have evolved very rapidly. Overall, our results indicate that foreleg length is an evolutionary highly labile, rapidly evolving trait that might enable *Rediviva* bees to respond quickly to changing floral resource availability.

1. Introduction

Since most pollinator species visit a broad range of host plant species (Bosch et al., 2009; Waser et al., 1996), morphological traits in pollinators are only rarely thought to have evolved in direct response to a specific host plant morphology (Feinsinger, 1983; Vázquez and Aizen, 2004). However, specialist pollinators that are highly dependent on a particular host taxon might exhibit remarkably bizarre morphological adaptations to their host plants. For example, some fly species within the Nemestrinidae and Tabanidae have developed an extremely elongated proboscis in order to obtain nectar from the long, narrow, tubular flowers of their host plants (Goldblatt and Manning, 2000). As flies with tongues matching the tubes of their hosts are likely to gain more floral reward, morphological adaptation to the host is probably associated with a fitness advantage (Pauw et al., 2009). However, we note that the phenotypic adaptations in the bee may not necessarily translate into fitness benefits and enhanced pollination of the plant.

Several bee taxa have developed specialised pilosity for collecting pollen. Central European bees of various families have convergently evolved highly specialised facial hair adapted to gather pollen from nototribic flowers in the families Lamiaceae and Scrophulariaceae (Müller, 1996). Other bees collecting pollen from the narrow flowers of Boraginaceae and Primulaceae have a highly modified leg and mouthpart pilosity for effectively extracting pollen from the hidden anthers of their host-plants (Muller, 1995). Particularly fascinating are adaptations of bees for collecting floral oil (Buchmann, 1987). Oil collecting behaviour has been documented in (i) the tribes Centridini, Tetrapedini, Ctenoplectrini and Tapinotaspini of the family Apidae (Buchmann, 1987; Cocucci et al., 2000; Houston et al., 1993; Neff and Simpson, 1981; Steiner and Whitehead, 2002) and in (ii) two of the genera of Melittidae: *Macropis* and *Rediviva* (Michez et al., 2009).

While the majority of bees are adapted to oil collection by possessing highly specialised hairs on either their legs or abdomen (Buchmann, 1987), females of the melittid genus *Rediviva* have evolved

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http://dx.doi.org/10.1016/j.ympev.2017.07.025
Received 28 December 2016; Received in revised form 22 June 2017; Accepted 26 July 2017
Available online 27 July 2017
1055-7903/

remarkably long forelegs for accessing floral oils within the elongate floral spurs of their principal host-plants (Vogel, 1974, 1984; Steiner and Whitehead, 1990, 1991). Apart from *Rediviva*, elongated forelegs have only been described for *Centris hyptidis* (Apidae) and *Tapinotaspis* species (Apidae) that regularly visit *Angelonia* (Scrophulariaceae, Machado et al., 2002), *Nierembergia* (Solanaceae, Cosacov et al., 2008) and *Sisyrinchium* (Iridaceae, Cocucci et al., 2000). This suggests convergent evolution of long legs for the purpose of oil collection.

In order to collect floral oil, a female *Rediviva* inserts its forelegs into the *Diascia* floral spurs, rubs against the spur walls that are covered with oil-secreting trichome elaiophores, and absorbs the oil with specialised hairs on the tarsal segments (Buchmann, 1987; Steiner and Whitehead, 1988; Vogel, 1984). The oil is then transferred to the bee's hindlegs (Vogel, 1984) and transported to the nest, where it serves as larval food and probably in brood cell lining (Pauw, 2006; Kuhlmann, 2014). During oil collection, pollen attaches to a species-specific part of the bee's body (Pauw, 2006; Waterman et al., 2011). About half of the *Diascia* species described are self-incompatible and highly dependent on *Rediviva* bees for reproduction (Steiner and Whitehead, 1988). Flowers of other Scrophulariaceae (*Alonsoa*, *Colpias*, *Hemimeris*), Iridaceae, Stilbaceae (*Anastrabe*, *Bowkeria, Ixianthes*) and Orchidaceae also serve as sources of oil for several *Rediviva* species (Kuhlmann and Hollens, 2015; Manning et al., 2002; Manning and Brothers, 1986; Pauw, 2006, 2005; Waterman et al., 2011; Whitehead et al., 2008; Whitehead and Steiner, 2001, 1996) and some of these plant species seem to depend solely on *Rediviva* for pollination (Steiner and Whitehead, 2002, 1991). In addition, all *Rediviva* species visit a range of local flowers for pollen or nectar (Whitehead et al., 2008; Whitehead and Steiner, 2001) and thereby likely pollinate them. From a conservation perspective, *Rediviva* bees are therefore probably of great importance for maintaining plant diversity in southern Africa (Pauw and Bond, 2011; Pauw and Hawkins, 2011).

Female *Rediviva* species vary in foreleg length from 6.5 mm (*R. albifasciata*) to an extreme of up to 26 mm (*R. emdeorum;* Whitehead and Steiner, 2001). Similarly, *Diascia* host species also vary in floral spur length, and significant covariation has been demonstrated between the bee's leg length and the plant's spur length for some *Rediviva* species and their *Diascia* hosts, indicating trait matching between the two taxa (Steiner and Whitehead, 1991, 1990, 1988); this is suggestive of high selective pressures on the interacting traits. The relationship is nonetheless not straightforward since many *Rediviva* species do not exclusively use just one *Diascia* host species for oil collection (Pauw, 2006; Steiner and Whitehead, 2002, 1988). Leg length of individual *Rediviva* might thus reflect adaptation to spur length of the local *Diascia* community (H. Hollens, pers. obs.). Such diffuse co-evolutionary associations might result in complex evolutionary dynamics and accelerated evolution of the underlying interaction traits, i.e. spur and leg length. Morphological adaptation to oil-collection on different host plants is also reflected in the foreleg pubescence of *Rediviva* females since oil presentation may vary across plant species (e.g. open or in coated droplets), requiring a range of specifically adapted bee pilosity types (Kuhlmann and Hollens, 2015).

Phenotypic specialisation such as the evolution of long legs might correlate with ecological specialisation as a specialised phenotype can limit the number of resources (or partners in the case of pollination) that can be used (Ollerton et al., 2007). For example, elongated feeding appendages such as an extended proboscis or front legs might be optimised for extracting rewards from deep flowers while being too unwieldy for accessing rewards from shallow flowers (Hollens et al., 2016; Pauw et al., 2009). If long front legs in *Rediviva* are also associated with a higher degree of ecological specialisation, measured as the number of interaction partners, this would suggest that leg elongation might play an essential role in diversification and speciation processes.

In this study we focus on the origins and evolution of leg length in *Rediviva*. In order to investigate how fast and how often long legs evolved within *Rediviva*, we first inferred the phylogenetic relationships within the genus *Rediviva* by sequencing six nuclear genes and one mitochondrial gene and reconstructing tree topologies using Bayesian approaches. We then mapped leg length onto the hypothesised phylogeny and estimated the tempo of leg length evolution. We also mapped other characteristics potentially associated with leg length variation, such as foreleg pilosity, bee distribution (winter vs. summer rainfall area), and the use of *Diascia* hosts, onto the phylogeny and tested if these traits are related to the evolution of leg length. Moreover, we also investigated if long legged bees are more specialised than short legged species. Our study not only provides the first thorough phylogeny for the genus *Rediviva* but is also the first to address the evolution of leg length across *Rediviva* bees.

2. Materials and methods

2.1. Phylogenetics and evolution of Rediviva

2.1.1. Taxon sampling and sequencing

Rediviva belongs to the family Melittidae, a small, relictual family that forms the sister group to all other extant bee families based on molecular data ((Brady et al., 2011; Branstetter et al., 2017; Hedtke et al., 2013; Kahnt et al., 2015; Peters et al., 2017). Melittidae is estimated to have originated in the late Cretaceous (Cardinal and Danforth, 2013; Branstetter et al., 2017; Peters et al., 2017). The monophyly of Melittidae has been under dispute for a long time since morphological synapomorphies are lacking and molecular studies either fail to support or only weakly support monophyly (Danforth et al., 2006a, 2006b, 2013; Michener, 2007). However, recent studies were able to provide substantial support for a monophyletic Melittidae (Branstetter et al., 2017; Hedtke et al., 2013). *Rediviva* might also be rendered paraphyletic due to the inclusion of the closely related genus *Redivivoides* (Michez et al., 2009).

The genus *Rediviva* is restricted to southern Africa, as are the approximately 73 species of its host plant genus *Diascia* (Steiner, 2011). Hitherto, 26 species of *Rediviva* have been described, with the majority (15) occurring in the winter rainfall area in the west of South Africa (Whitehead and Steiner, 2001; Whitehead et al., 2008; Kuhlmann, 2012a). This area is largely congruent with the Greater Cape Floristic Region (GCFR), a global biodiversity hotspot (Myers et al., 2000). Within the winter rainfall area, *Rediviva* species are often restricted to only a small geographic area, maybe due to the distribution of host plants or special climatic requirements (Kuhlmann, 2009). The summer rainfall area encompasses a larger part of southern Africa but includes only 11 *Rediviva* species that live predominantly in grassland habitats (Whitehead et al., 2008; Kuhlmann, 2012a).

In this study, we sampled a total of 66 specimens across South Africa (see Suppl. Table 1), comprising 19 species of *Rediviva* and 3 out of 7 described species of the closely related genus *Redivivoides* (Kuhlmann, 2012b). We also included the following previously sequenced outgroups: 24 *Melitta* species, three *Macropis* species and *Promelitta alboclypeata* (see Suppl. Table 1). DNA of *Rediviva* and *Redivivoides* specimens was extracted either using a phenol-chloroform (Danforth et al., 1999) or a high-salt-extraction protocol (Paxton et al., 1996). We amplified partial mitochondrial cytochrome *c* oxidase subunit I (COI), the nuclear ribosomal 28S (D2-D3 region) gene, and the following five nuclear protein-coding genes: elongation factor-1α F2 copy (EF-1α), wingless (Wg), long-wavelength rhodopsin RH1 (Ops), sodium-potassium ATPase (NaK), and RNA polymerase II (Pol II). Polymerase chain reactions (PCRs) were carried out following the protocol of Danforth et al. (2011) and using either Promega Go-Taq Flexi or G2 DNA polymerase (http://www.danforthlab.entomology.cornell.edu/resources.html). When nonspecific amplicons were amplified by PCR, we reduced $MgCl_2$ to 1 mM. PCR conditions as well as primer-specific annealing temperatures and primer sequences are given in Suppl. Table 2. PCR products were purified using either the ExoSAP-IT® PCR Product Cleanup kit (Affymetrix) or the QIAquick PCR Purification Kit

(QIAGEN) following the manufacturer's instructions. Purified PCR products were sequenced in both directions on an Applied Biosystems Automated 3730xl DNA Analyzer (Thermo Fisher Scientific; Waltham, Massachussetts, USA) at the Cornell University Biotechnology Resource Center or by GATC-Biotech (Constance, Germany) sequencing service. Accession numbers are provided in Suppl. Table 1.

2.1.2. Sequence processing and phylogenetic analyses

Sequence data were checked for identity by a BLAST search against the NCBI GenBank database and then trimmed and edited in SEQUENCHER v. 5.2.4 (Gene Codes Corporation, Ann Arbor, MI, USA) or Geneious v. 7.1.7 (Kearse et al., 2012). In addition to our newly generated sequences we added previously published sequences for three species of *Rediviva* and three species of *Redivivoides* (Dellicour et al., 2014, Suppl. Table 1) to our data set. Individual gene sequences were aligned using the online version of MAFFT v. 7 (Katoh and Standley, 2013) and inspected for an open reading frame and manually edited in Mesquite v. 3.03 (Maddison and Maddison, 2008). Intron/exon boundaries within EF-1α and long-wavelength rhodopsin were inferred by alignment against the *Apis mellifera* genome sequence. Since intron sequences could be aligned without major difficulties, they were included in the final data set. Only highly ambiguous sequence regions of 28S (15 nucleotide sites overall) had to be excluded from final analyses. Concatenation of individual gene alignments to a supermatrix was performed in SequenceMatrix v. 1.7.9 (Vaidya et al., 2011). In order to reduce alignment ambiguities when comparing highly variable regions between distantly related taxa, we excluded all *Macropis* species and *Promelitta alboclypeata*, realigned and reran analyses. The final supermatrix data set without the outgroup sequences comprised 5370 aligned nucleotide sites. PartitionFinder v. 1.1.1 (Lanfear et al., 2012) was used to determine the best partitioning scheme and nucleotide substitution models. Phylogenetic analyses were then run in MrBayes v. 3.2.3 (Ronquist and Huelsenbeck, 2003) via the CIPRES Science Gateway (Miller et al., 2010), either under the models suggested by PartitionFinder (PF analysis) or under the reversible-jump procedure (RJ analysis). For both approaches, two runs with four Markov chains each were conducted under default options for 10,000,000 generations with a burn-in of 25% of the samples and sampling every 1000 generations. Convergence of the Markov chains was confirmed in Tracer v. 1.5 (Rambaut and Drummond, 2009) by a combined effective sample size (ESS) > 200.

2.1.3. Dating of divergence events

In order to determine divergence times of key clades within the phylogeny, we included the *Macropis* and *Promelitta* specimens and calibrated our phylogeny using fossil information. There are only few melittid fossils available for calibration (Michez et al., 2012): *Eomacropis glaesaria* (42 MYA; Engel, 2001), *Macropis basaltica* (23 MYA; Zhang, 1989; Michez et al., 2007) and *Paleomacropis eocenicus* (53 MYA; Michez et al., 2007). *Eomacropis glaesaria* and *P. eocenicus* are probably stem group fossils of Macropidini. *Macropis basaltica* probably represents a stem group of *Macropis*. We thus used 53 MYA (mean = 5 MYA, standard deviation: 1 MYA, offset: 53 MYA) as a prior to date the split between Macropidini and Melittini and 23 MYA (mean = 5 MYA, standard deviation: 1 MYA, offset: 23 MYA) for the split between *Promelitta* and *Macropis* using a lognormal distribution.

We partitioned our data set according to PartitionFinder analysis and ran analyses in BEAST 2 (Bouckaert et al., 2014), applying the Reversible Jump procedure (via the RBS plug-in) to each partition. An uncorrelated lognormal relaxed clock model, which allows for variation in substitution rates between branches, was employed as this model showed the highest likelihood of all clock models according to a Bayes factor tests in Tracer ($\log_{10}$ Bayes factors of lognormal relaxed clock model versus random local clock = 366, versus relaxed exponential clock = 30 and versus strict clock = 228). Substitution rates were estimated relative to each other with the second partition, which includes the relatively conserved first coding positions of all nuclear genes apart from opsin, set as 1. We also used a Yule tree prior assuming a constant speciation rate per lineage with no extinctions as it has been shown to be most appropriate for inferring phylogenetic relationships on the species level (Drummond et al., 2012). The birth rate of the tree and the mean of the branch rates under the uncorrelated lognormal relaxed clock model (ucldMean) were estimated using a gamma distribution with alpha = 0.001 and beta = 1000 to obtain a diffuse prior. All other parameters were left at default. We ran two MCMC chains for 100,000,000 generations, using a burn-in of 25% and sampling every 100,000 generation to ensure convergence, and combined the results of the different runs with LogCombiner (Bouckaert et al., 2014). A maximum clade credibility tree was calculated in TreeAnnotator (Bouckaert et al., 2014), and the median node ages are reported.

2.2. Evolution of leg length variation within Rediviva

2.2.1. Mapping of female foreleg length within Rediviva

We chose to map relative foreleg length rather than absolute foreleg length since larger sized bees will inherently show longer legs. Relative leg lengths ratios were calculated by using foretarsus length as a proxy for foreleg length and forewing length as a proxy for body size (sample size: 10–59 individuals per species). Most measurements were taken from the literature (Steiner, 2010; Steiner and Whitehead, 2002, 1990; Whitehead et al., 2008; Whitehead and Steiner, 2001, 1992). Additional measurements were taken for *Melitta*, *Redivivoides* and *Rediviva steineri*, *R. neliana* and *R. albifaciata* using the methods in Steiner and Whitehead (1990). Due to the availability of only a few, partially damaged *R. neliana* (N = 3), measurements for tarsus and wing length had to be taken from two different *R. neliana* populations. In the case of *R. saetigera* and *R. colorata*, we were unable to take measurements for tarsus and forewing length and could also not extract that information from the literature. Hence, we could not map the relative leg length onto the tree for these two species. For the discrete analyses we distinguished between long- and short-legged species, and refer to a bee as 'long-legged' if the forelegs are longer than the body length (Kuhlmann and Hollens, 2015). Hence, in our data set *R. micheneri*, *R. neliana*, *R. longimanus*, *R. macgregori*, *R. emdeorum* and *R. colorata* are defined as long-legged species.

Leg length was then mapped onto the phylogeny inferred by RJ in MrBayes as this topology also accounts for uncertainty in the placement of the clade comprising *R. macgregori*, *R. longimanus*, and *R. peringueyi* (hereafter referred to as clade C, see results). For mapping purposes we collapsed branches of individuals from the same species in R v. 3.2.4 (R Core Team, 2016) using the drop.tip function of the package geiger (Harmon et al., 2008). Mapping of continuous and discrete relative leg length ratios onto the phylogeny was performed using parsimony implemented in Mesquite v. 3.03.

To reconstruct foreleg length at ancestral nodes of the *Rediviva* phylogeny, we used an MCMC approach implemented in BayesTraits v. 2.0 (Pagel et al., 2004). We first computed the best fitting model for ancestral trait reconstruction using a continuous trait random walk model and then inferred relative leg length at major nodes of the *Rediviva* phylogeny using reversible jump MCMC with a hyper exponential prior. Apart from the rate deviation parameter of acceptance, which was set to 2.0, all other parameters were left at default. We ran analyses for 10,000,000 generations, sampling every 10,000 generations and discarded the first 25% of the output as burn-in.

2.2.2. Multiple independent versus a single origin of long legs

Given that the character mapping in Mesquite indicated several independent origins of long legs, we tested an unconstrained topological hypothesis versus an alternative hypothesis in which we forced all long-legged species into one monophyletic unit, i.e. having one origin. We calculated the Bayes factor by comparing twice the difference in the marginal likelihoods of the two hypotheses in MrBayes v. 3.2.3

(Ronquist and Huelsenbeck, 2003). Analyses were run using the stepping stone method and 50 steps with 196,000 generations within each step, summing to a total of 9,996,000 generations. The first 196,000 generations were discarded as initial burnin.

2.2.3. Phylogenetic non-independence of leg length variation patterns and modelling of leg length evolution

We tested for a phylogenetic signal of foreleg length, i.e. if closely related species are more similar to each other in terms of leg length than more distantly related taxa. We employed Pagel's λ as a measure of phylogenetic signal as it has been shown to outperform alternative measurements (Munkemüller et al., 2012). Pagel's λ is a measure of phylogenetic correlation defined as the transformation of the phylogeny that makes the trait data best fit a Brownian motion (BM) model, with a λ value of zero indicating phylogenetic independence (Freckleton et al., 2002). We calculated Pagel's λ (Pagel, 1999) for the log transformed data with the function pgls (package caper, Orme, 2013) in R v. 3.2.4 (R Core Team, 2016).

Since BM models might not be appropriate for traits assumed to be under strong selection (Butler and King, 2004), we also modelled the evolution of leg length with the R packages ape (Paradis et al., 2004) and nlme (Pinheiro et al., 2017) using a phylogenetic generalised least square (PGLS) approach (function gls) assuming the Ornstein-Uhlenbeck (OU) model (Hansen, 1997) (function corMartins). We checked results against a pure BM model (function corBrownian) in the PGLS approach.

As the evolution of phenotypic specialisation such as leg length variation in *Rediviva* might also be correlated with the evolution of ecological specialisation, we included specialisation, measured as the number of interaction partners, as a predictor in our models of leg length evolution while controlling for phylogenetic non-independence assuming a BM or OU model. Model comparisons were performed with the package MuMIn (Bartoń, 2016, function model.sel) based on the Akaike information criterion (AIC). Host record data for *Rediviva* species were extracted from the collection data of the South African Museum (Whitehead and Steiner, 3758 specimens, mainly captured on *Diascia*), the museum collection of the Stellenbosch University (240 specimens, including many captured on orchids) and from the literature (Steiner, 2010, 1989, 31 specimens collected from orchids).

Rediviva saetigera and *R. colorata* could not be included in these analyses since information on their relative leg lengths was missing. We also removed the *Melitta* outgroup and all three *Redivivoides* species as they do not collect oil.

2.2.4. Rate of leg length evolution and leg length dependent speciation rates

In order to determine the rate of leg length evolution and whether some clades show an accelerated evolution of this trait, we modelled change in (log) leg length along our time calibrated BEAST tree in BAMM v 2.5.0 (Rabosky, 2014). BAMM employs a reversible jump MCMC approach, automatically exploring the most suitable model(s) for the data (Rabosky, 2014). Markov chains were run for 10,000,000 generations, sampling every 10,000 generations and discarding the first 10% of the sample as burn-in. Appropriate priors were estimated beforehand with the package BAMMtools (Rabosky et al., 2014, function setBAMMpriors) in R. All other parameters were left as default. BAMMtools was then used to calculate the overall rate of leg length evolution (function getCladeRates) and the average rate of leg length evolution for individual clades (function getCladeRates) or species (function getTipRates) assuming a multi-rate BM model. For these analyses we excluded outgroups, *R. saetigera* and *R. colorata* due to lack of data.

Next we assessed whether an increase in leg length was associated with increased speciation (λ) by running a FISSE analysis (Fast, intuitive State-dependent Speciation and Extinction, Rabosky and Goldberg, 2017) in R. In the FISSE approach, a test statistic is computed that compares the distributions of branch lengths for lineages with and without a character state of interest. Significance is assessed by comparison to a null distribution generated by simulating character histories on the observed phylogeny. We ran 1000 simulations to generate a null distribution and also accounted for the proportion of non-sampled species in our analyses.

2.2.5. Correlated evolution between leg length and other potentially adaptive traits

We also mapped the following three discrete characters onto the MrBayes phylogeny in Mesquite, which we hypothesised might be of relevance for the origin of long forelegs in *Rediviva* and adaptation to a host plant: (1) the geographic distribution of *Rediviva* species (winter versus summer rainfall area), (2) species reported to visit *Diascia* (yes versus no; Whitehead and Steiner, 2001; Whitehead et al., 2008) and (3) foreleg pilosity type (type I, II, III or IV according to Kuhlmann and Hollens, 2015; see Supp. Fig. 1).

We tested for a significant correlated evolution between leg length and geographic distribution or leg length and *Diascia* visitation using the Pagel94 test for discrete character evolution (Pagel, 1994) in Mesquite, employing 1000 Monte Carlo simulation replicates. The Pagel94 test compares the ratio of the likelihoods of a model of independent evolution of the two characters under study and a model of correlated character evolution. As the initial analyses suggested a link between *Diascia* visitation behaviour and the presence of absorptive hairs on the forelegs, we also tested for a relationship between these two traits. Since the Pagel94 test is only suitable for binary characters, we coded each individual pilosity type as discrete (present versus absent) and individually tested if the respective pilosity type was correlated with leg length.

2.3. Reconstruction of ancestral biogeography

We reconstructed the ancestral geographic distribution (winter versus summer rainfall area) distribution of *Rediviva* using the program Reconstruct Ancestral States in Phylogenies (RASP) v3.2 (Yu et al., 2015) employing four different algorithms and assessing the degree of congruence between these methods: statistical dispersal-vicariance analysis (S-DIVA; Yu et al., 2010), Lagrange dispersal-extinction-cladogenesis (DEC; Ree and Smith, 2008), Bayes-Lagrange statistical DEC (S-DEC; Beaulieu et al., 2013) and Bayesian Binary MCMC (BMM). To account for uncertainty in phylogenetic reconstruction we used the 2000 trees calculated in the two BEAST runs as input, collapsing branches of individuals belonging to the same species and removing all outgroups, as recommended (Yu et al., 2012). All analyses were run under default settings.

3. Results

3.1. Phylogenetics and evolution of Rediviva

Our final data set consisted of seven genes from 19 *Rediviva* species, three *Redivivoides* species and 28 melittid outgroups. Bayesian analyses of our data set using either RJ or PF models in MrBayes or RJ in BEAST resulted in high support for many nodes, including the position of the majority of the long-legged species (Fig. 1). All *Rediviva* species were suggested to be monophyletic except for two cases: *R. nitida* and *R. peringueyi*. *Rediviva nitida* nested within *R. micheneri* in the RJ analyses in MrBayes (posterior probability = 0.99) and rendered the latter paraphyletic. However, BEAST analyses run under the same RJ procedure as well as MrBayes analyses under PF models strongly supported the monophyly of *R. micheneri* (Fig. 1). BEAST analyses also returned *R. peringueyi* as paraphyletic due to the inclusion of *R. longimanus* but support values were relatively low (0.78 in Fig. 1). Since there was only low support for considering *R. micheneri* and *R. peringueyi* as paraphyletic taxa, because the number of of genes sequenced and the number of individuals per species was too small to reliably reject (or

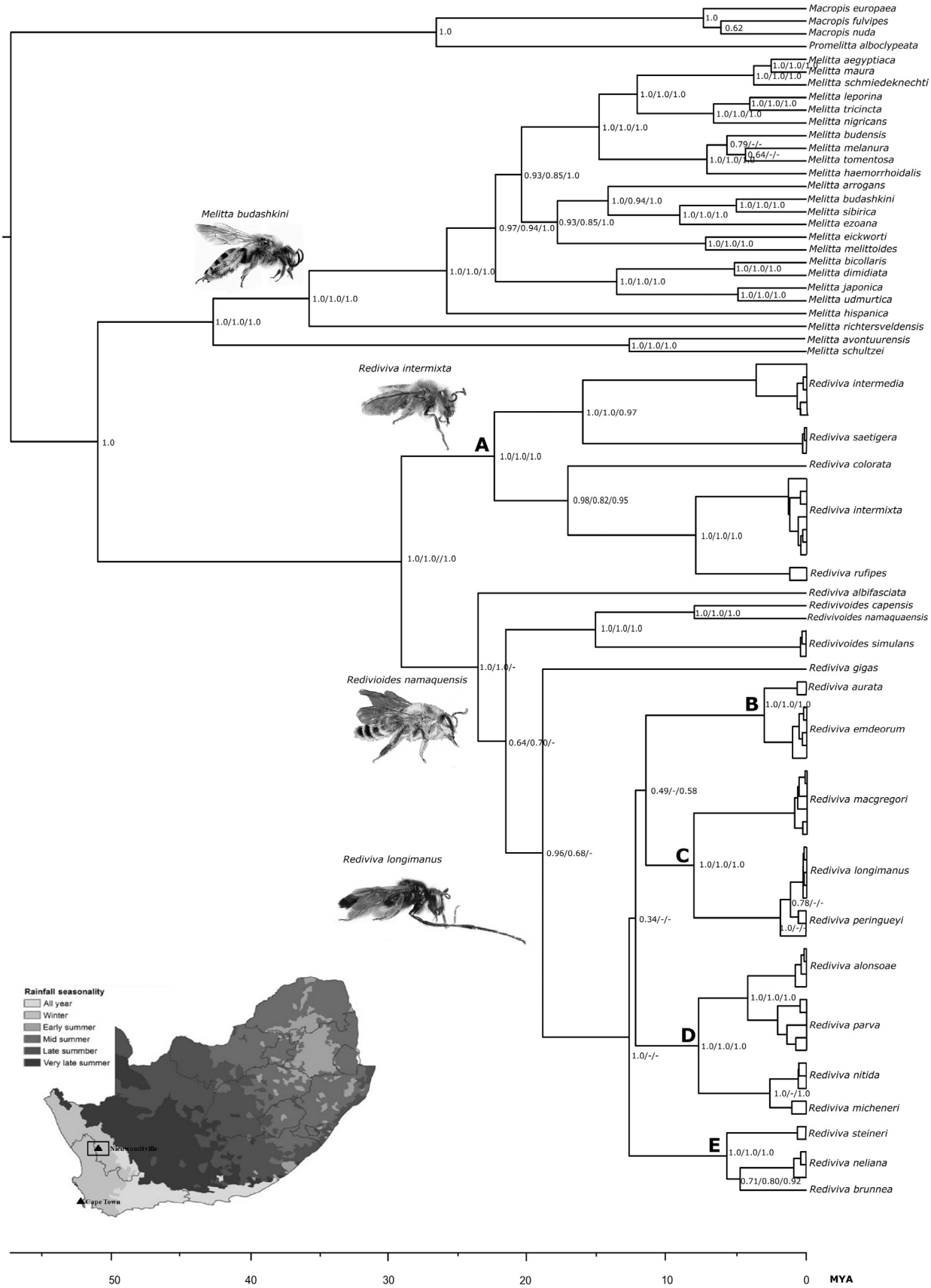

Fig. 1. Phylogeny of the genus *Rediviva*. Several *Melitta* species as well as two *Macropis* and one *Promelitta* species served as outgroups. For ease of representation, posterior probabilities for nodes are given only for inter- but not intraspecific relationships and correspond to reconstructions in BEAST (first value) or MrBayes using a reversible jump approach (second value) or models according to PartitionFinder (third value). Dates for divergence events were calculated in BEAST using fossil information for the split between Macropidini and Melittini (53 MYA) and for the split between *Promelitta* and *Macropis* (23 MYA), and are indicated below the tree topology in MYA.

support) monophyly, we continued treating both species as monophyletic units in further analyses.

Since several clades were consistently recovered and supported by a posterior probability of 1.0 in all three analyses, we defined the following five major clades of *Rediviva*: clade A, comprising *R. colorata*, *R. intermixta*, *R. rufipes*, *R. intermedia* and *R. saetigera*; clade B, containing *R. aurata* and *R. emdeorum*; clade C, including *R. longimanus*, *R. peringueyi* and *R. macgregori*; clade D, consisting of *R. alonsoae*, *R. parva*, *R. micheneri*, *R. nitida*, and clade E, containing *R. brunnea*, *R. steineri* and *R. neliana*. We could have also distinguished between only two clades, i.e. our clade A versus all others (B to E) since this split also received highest support across all three analyses. However, the ecological traits we analysed (distribution area and host plant spectrum) are mainly shared between all members within our five clades, making each clade relatively homogeneous, and also biologically more reasonable than just two very heterogeneous clades.

In all analyses, *Redivivoides* nested within *Rediviva* and rendered *Rediviva* paraphyletic, but the exact position of *Redivivoides* varied among analyses and neither alternative received high support values (posterior probability max. 0.7, Fig. 1). Another consistent finding was the placement of clade A as sister to all other clades. Phylogenetic relationships among the other major clades were unstable across the different analyses and could not be resolved. While both analyses in MrBayes (RJ and PF) supported a sister group relationship between D and E, the analysis in BEAST suggested a sister group relationship between D and the two other clades, B and C. The phylogenetic relationship among the five clades and the other two *Rediviva* species (*R. albifasciata* and *R. gigas*) could also not be resolved with high certainty.

Dating of divergence events suggested an origin of the *Rediviva* clade at 28.9 MYA (Fig. 1, 95% highest posterior density, HPD, interval = 19.2–40.5). The five *Rediviva* crown groups seem to have arisen in a timeframe of 22–13 MYA. *Redivivoides* likely originated 19 MYA (Fig. 1, 95% HPD interval = 7.8–22.1).

3.2. Mapping of female foreleg length and origins of long legs within Rediviva

Mapping of traits onto the *Rediviva* phylogeny indicated several independent origins of long legs within *Rediviva*. Although long-legged bees (i.e. with legs longer than the body size) tend to occur mainly in clades B to E (Fig. 2, Supp. Fig. 2), one other long-legged species, *R. colorata,* belongs to clade A. According to the most parsimonious explanation provided by the Mesquite analyses, long legs arose at least five times independently in the evolution of *Rediviva*: *R. colorata*, *R. emdeorum*, *R. neliana*, *R. micheneri* and a potentially shared origin for *R. macgregori* and *R. longimanus*, with only one reversal back to a short legged condition. Alternatively, long legs might have two evolutionary origins: one in *R. colorata* and one for the whole BCDE clade. However, this would require 7 evolutionary reversals to short-legged condition within the BCDE clade, namely for *R. aurata*, *R. peringueyi*, *R. steineri*, *R. brunnea*, *R. parva*, *R. alonsoae* and *R. nitida*. Since we lack information about the relative leg lengths for *R. saetigera* and *R. colorata*, we could not distinguish between these two hypotheses nor could we calculate the ancestral leg length for the common ancestor of all *Rediviva* and *Redivivoides* species using BayesTraits. Nevertheless, parsimony reconstruction in Mesquite indicated that the common ancestor of *Rediviva* and *Redivivoides* did not possess elongated forelegs (Fig. 2).

Assuming the ancestor of *R. colorata* to be the first taxon exhibiting long legs, long legs arose in the last 17 million years (Fig. 1, 95% HPD interval = 9.9–26.8 MYA). Hypothesising the first occurrence of long legs for the common ancestor of the BCDE clade would necessitate an even shorter evolutionary history for leg length elongation of 12 MYA (95% HPD interval = 7.6–18.2 MYA). Thus 12 MYA seems to be the lower bound for an age estimate of the first origin of long legs. This is because ancestral state reconstruction in BayesTraits indicated long legs to be already present in the ancestors of the BCDE clade (Suppl. Fig. 2).

3.3. Multiple independent versus a single origin of long legs

According to our hypothesis testing in MrBayes, we found the mean marginal log likelihood of a topology forcing all long-legged *Rediviva* species to share a common ancestor to be −29377.20. The mean marginal log likelihood of an unconstrained topology was −28763.09. Thus, the Bayes factor of the two hypotheses is 1228.22. According to Kass and Raftery (1995) a Bayes factor ≥10 represents very strong support for the alternative hypothesis. Hence, our data strongly reject the hypothesis of a single origin of long legs and the monophyly of long-legged *Rediviva* species.

3.4. Controlling for phylogenetic non-independence of leg length and modelling variation in leg length

When testing for a phylogenetic signal of leg length, we could not detect a signal with Pagel's λ based on a BM model (λ = 0.0, lower bound p = 1.0). However, when we modelled leg length evolution employing the OU model (AIC = −18.0, Table 1), it fit the data much better than a pure BM model (AIC = −4.7, Table 1), which would be expected for traits experiencing certain modes of selection. Based on the OU model, we estimated the selection-strength parameter α, to be 8.96.

Incorporating ecological specialisation did not improve the fit of our models of leg length evolution (specialisation-OU model: AIC = −16.6, specialisation-BM model: −3.0, Table 1). Thus, phenotypic specialisation as exhibited by the evolution of elongated front legs in *Rediviva* females seems not to be correlated with ecological specialisation in the taxon.

3.5. Rate of leg length evolution and leg length dependent speciation rates

Calculation of the rate of leg elongation suggested that branch-specific evolutionary rates for several *Rediviva* species were markedly higher than the average for the *Redivivoides* + *Rediviva* clade. The rate of foreleg length evolution accelerated markedly in the BCDE clade, containing the majority of long-legged bees, and further increased in the BC clade, which contains the most extreme taxa in terms of relative leg length: *R. emdeorum* and *R. longimanus* (Supp. Fig. 3). Not surprisingly, branch-specific rates of leg length evolution were also highest for these two species.

However, we could not find a significant difference in diversification rates between long-legged (λ_1 = 0.12 species per million years) and short-legged (λ_0 = 0.09 species per million years) *Rediviva* species (p = 0.88).

3.6. Correlated evolution between leg length and other traits of adaptive potential

Leg length in *Rediviva* is assumed to be closely linked to oil collection from *Diascia* flowers. We inferred that the ancestor of *Rediviva* did not visit *Diascia* (Fig. 2). Apart from *R. saetigera*, *R. albifasciata*, *R. gigas* and *R. alonsoae*, all other extant *Rediviva* species were recorded to use *Diascia* as an oil resource (Fig. 2). Although the Pagel94 test did not reveal a correlation between leg length and *Diascia* visitation (p = 0.58), *Diascia* visitation was significantly correlated with the possession of absorptive hairs on the forelegs (p ≤ 0.01). Similarly to *Redivivoides*, neither *R. gigas* nor *R. saetigera* visit *Diascia* nor do they exhibit absorptive hairs on the foretarsi. These two *Rediviva* species are therefore classified as a separate pilosity type category (type IV).

Apart from the link to *Diascia* visitation, pilosity types seem to be randomly distributed over the phylogeny, with sister species often varying in their pilosity type (Fig. 2). There also seems to be no relationship between pilosity and leg length because the Pagel94 test was not significant (p ≥ 0.05) for any of the four pilosity types tested. Long-legged *Rediviva* species may exhibit type I or II pilosity while short legged species show all four pilosity types (Fig. 2). All four pilosity

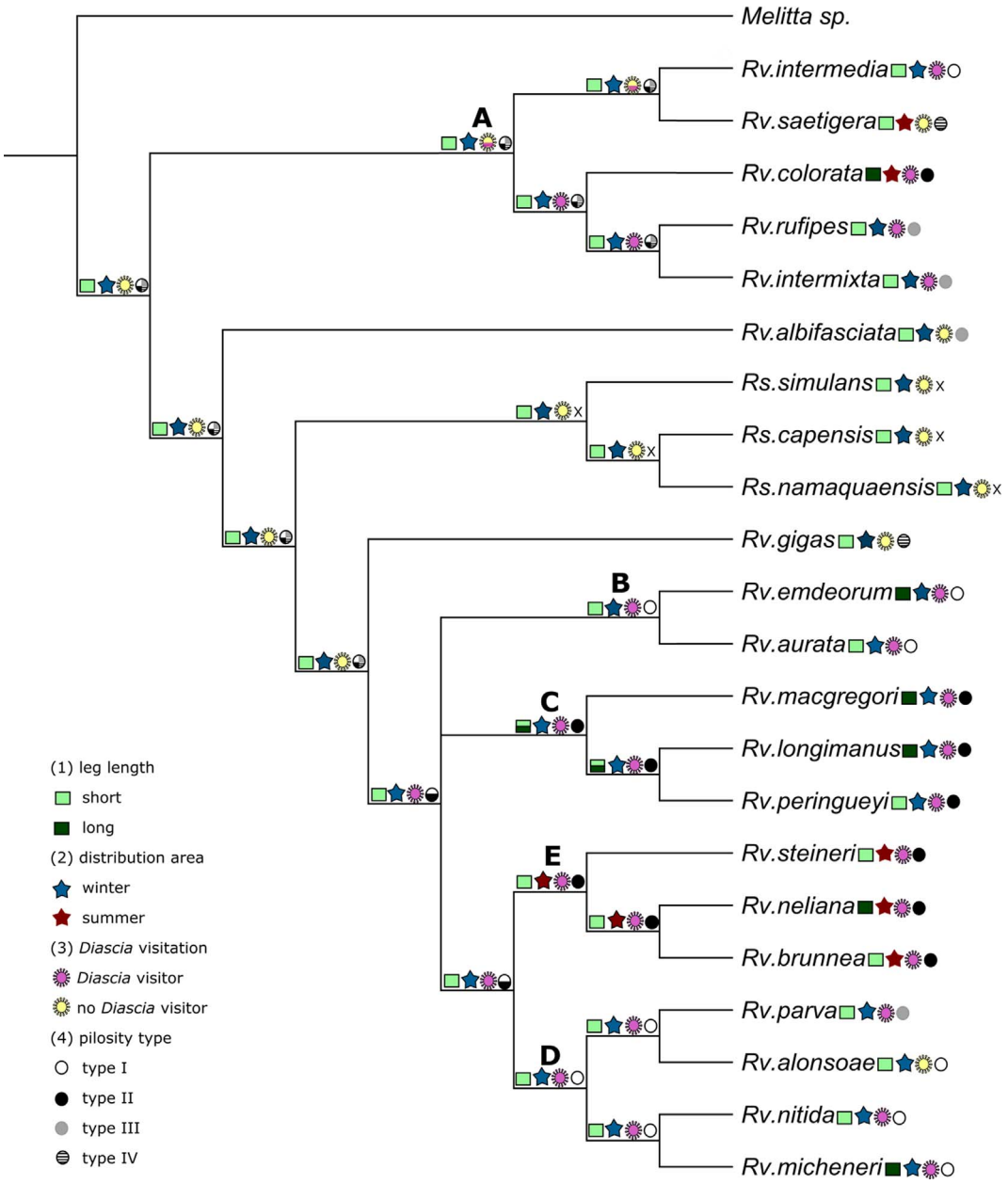

Fig. 2. Mapping of four discrete traits of *Rediviva* using maximum parsimony implemented in Mesquite: (1) leg length (light green box = short, dark green box = long), (2) winter or summer rainfall region (blue star = winter, red star = summer), (3) *Diascia* visitation (yellow flower = no, pink flower = yes) and (4) pilosity type (white circle = type I, black circle = type II, grey circle = type III, dashed circle = type IV, according to Kuhlmann and Hollens (2015)). Missing information for traits is indicated by an x. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Results of the phylogenetic generalised least square regressions (PGLS) including or excluding the effect of host specialisation ("host") and assuming either the Brownian motion (BM) or Ornstein-Uhlenbeck (OU) model.

Model	Intercept	Host coefficient	df	logLik	AIC
OU	−0.37	–	3	12.00	−18.00
OU & host	−0.39	0.00	4	12.11	−16.20
BM	−0.44	–	2	4.34	−4.70
BM & host	−0.40	0.00	3	4.68	−3.40

types seem equally likely as the ancestral state, based on parsimony reconstruction.

The relationship between leg length and distribution area (today's winter or summer rainfall area) of species was also not significant ($p = 0.82$). Out of six long-legged *Rediviva* species, two are found in the summer rainfall area while the remaining four occur in the winter rainfall area (Supp. Fig. 4).

3.7. Ancestral biogeographic distribution

Based on character mapping in Mesquite the *Rediviva* ancestor originated in the winter rainfall area (Fig. 2). However, historical biogeographic reconstruction in RASP provided strong support for an origin in the winter rainfall area only under the BBM algorithm (98% in the winter rainfall area) while all other approaches allowed for the possibility that the *Rediviva* ancestor was already distributed in both areas: S-DIVA: 50% in both areas versus 50% exclusively in the winter rainfall area, S-DEC: 56% in both areas and 44% exclusively winter rainfall area, DEC: 60% in both areas and 40% exclusively winter

rainfall area (Supp. Fig. 5).

Most extant *Rediviva* species occur in the winter rainfall area, whereas species of the summer rainfall area belong to either clade E (*R. neliana*, *R. brunnea*, *R. steineri*) or clade A (*R. saetigera*, *R. colorata*, Supp. Fig. 4). The most likely scenario explaining the current distribution pattern is the dispersal of the ancestor of *R. gigas* and the BCDE clade to the summer rainfall area followed by a vicariance event experienced by the ancestor of the E clade to the other clades (S-Diva: p = 1.0, DEC: p = 1.0). Similarly, the current distribution of the two extant summer rainfall species of the A clade, *R. saetigera* and *R. colorata*, is probably best explained by the migration of the ancestor of the clade A to the summer rainfall area (S-DIVA: p = 0.5, DEC: p = 0.76, S-DEC: p = 0.52), followed by two vicariance events experienced by the ancestors of *R. saetigera* and *R. colorata* and their respective sister group (S-DIVA: p = 1.0, DEC: p = 1.0, S-DEC: p = 0.87).

4. Discussion

Overall, our study is not only the first thorough analysis of phylogenetic relationships within the bee genus *Rediviva* but also reveals great flexibility in the evolution of an ecologically important trait, foreleg length. Our analyses indicated variability in foreleg length, even between sister species, and at least two independent origins of long legs. Interestingly, the first origin of long legs seems to be markedly younger (12–17 MYA) than the origin of the taxon *Rediviva* itself (29 MYA), as the ancestor of all *Rediviva* was inferred to be a short-legged species which did not visit *Diascia*.

Although we are missing seven of the 26 described *Rediviva* species, we do not expect that non-sampled species have a strong impact on our major findings. We might expect subtle change in the topology of our phylogeny and definition of our clades after including the missing taxa. Our main finding of strong support for several independent origins of long legs would not change by the inclusion of additional species but they would rather strengthen the rejection of a single origin by potentially revealing additional origins of long legs. Moreover, we might still expect to see a high rate of evolutionary change in leg length after the inclusion of currently non-sampled species as we already detected a strong signal in our data, which is unlikely to disappear after inclusion of additional taxa. However, we cannot rule out the possibility that we might detect a correlation between leg length evolution and one or more of the other variables investigated, i.e. distribution area, pilosity type, ecological specialisation and *Diascia* visitation behaviour, after adding the seven missing species.

4.1. Rediviva phylogenetics

Our phylogenetic analyses provided strong support for five clades within *Rediviva*. Phylogenetic affinities between these clades could not be resolved unambiguously and require further investigation. The genus *Redivivoides* was consistently recovered as a monophyletic taxon nested within *Rediviva*, rendering the latter paraphyletic, a finding supported by a previous phylogenetic study (Michez et al., 2009). Some of the analyses suggest *R. peringueyi* and *R. micheneri* to be paraphyletic since *R. longimanus* or *R. nitida* nested within them, respectively. Nevertheless, in both cases only one of the three phylogenetic analyses supported paraphyly of the respective species, suggesting that the molecular signals of our marker genes were not sufficient to resolve speciation events that are younger than 2.6 MYA (i.e. divergence time of *R. nitida* and *R. micheneri*) and that took place within a short time frame of a few thousand years.

4.2. Multiple origins of long legs and rapid evolution of leg length variation

Long legs seem to have arisen not once but at least twice in the evolutionary history of *Rediviva*. This is strongly supported not only by our mapping analyses but also by hypothesis testing in MrBayes. It is also reflected in the lack of a phylogenetic signal for leg length. The lack of phylogenetic signal for leg length variation based on a Brownian motion model of evolutionary drift indicates that phylogenetic relationships and drift alone are unlikely to account for the evolution of long legs in *Rediviva*. Thus, other evolutionary forces such as selection might have shaped the trait. Under the OU model, the estimated α parameter was 8.02. Since a value for $\alpha > 2$ is considered high (Beaulieu et al., 2012), it suggests that leg length has experienced strong selection in response to variation in the length of host plant spurs (Steiner and Whitehead, 1991, 1990).

Our analyses suggest leg length is a highly labile trait that evolves very rapidly. Trait mapping revealed that even sister species can be highly different in their mean leg length (e.g. *R. emdeorum* and *R. aurata*) and calculations of the rate of leg length evolution indicated an acceleration in the clade with the majority of long-legged bees. Although we could not detect an association between long legs and diversification in the FISSE analysis, we cannot completely rule out the possibility that leg elongation might drive speciation. Since the genus *Rediviva* comprises only 26 species, of which 19 were included in our study, we necessarily had low statistical power. Thus, more thorough genetic analyses that directly tackle the genetic basis of leg length variation and investigate levels of gene flow between populations differing in leg length are required to test the idea that leg length evolves rapidly and might drive speciation.

4.3. Rediviva leg morphology and adaptation to Diascia host plants

We could not find support for correlated evolution between long legs and use of *Diascia* hosts, possibly because not all *Diascia* are long-spurred. However, we detected a statistically significant relationship between *Diascia* visitation and the presence of absorptive hairs on foretarsi. In contrast to *Diascia* visitors, bees that do not use *Diascia* as host-plants, like *R. gigas* and *R. saetigera*, largely lack absorptive hairs on the tarsi (pilosity type IV), indicating that the evolution of absorptive hairs and *Diascia* visitation might be coupled and might represent derived characteristics. In a separate study, we specifically test whether leg length evolution in *Rediviva* is an adaptation to spur length variation in its host plants (Pauw et al., 2017; in review).

Studies in other plant-pollinator systems have also shown that the morphology of the pollinator is tightly linked to the host plant and might be of great relevance for the fitness of each interaction partner. In the long proboscid fly *Prosoeca ganglbaueri* (Nemestrinidae), tongue length covaries with the tube length of its primary food resource, *Zaluzianskya microsiphon* (Anderson and Johnson, 2007), as well as the mean tube length of the local host plant guild (Anderson and Johnson, 2009). Moreover, selection experiments with the main host *Z. microsiphon* suggested that tube length is adaptive and probably arose via co-evolution with *P. ganglbaueri* (Anderson and Johnson, 2007). Similar studies of the long-proboscid fly *Moegistrorhynchus longirostris* showed that tongue length of the pollinator is significantly correlated with tube length of its guild of host plants, and that the match between these traits affects the fitness of both interaction partners, indicative of reciprocal selection (Pauw et al., 2009).

The evolution of foreleg length in *Rediviva* is probably tightly linked to their oil collection behaviour and thus the evolution of spur length in their *Diascia* hosts (Steiner and Whitehead, 1991, 1990). We found strong support for a sister group relationship of *Rediviva* to the non-oil collecting genus *Melitta* rather than to the oil-collecting genus *Macropis*, a view supported by Michez et al. (2009), who suggested oil-collecting behaviour arose two times independently, once in *Rediviva* and once in the common ancestor to *Macropis* and *Paleomacropis*. Our study supports this scenario and also allows for the possibility that oil-collecting behaviour might not have been present in the common ancestor of *Rediviva* and *Redivivoides* since it was inferred to be a short-legged bee that did not visit *Diascia*. *Rediviva* generally seems to possess great potential for adaptation to and switching between host plants based on

the scattered distribution of pilosity types and long legs across the *Rediviva* phylogeny, which indicates rapid evolution of foreleg traits that are primarily involved in the interaction with the host plant or, alternatively, a generally high evolvability (Hansen, 2006) of the taxon.

Ecological flexibility is indeed reflected in the wide spectrum of oil hosts used by different *Rediviva* species. Even individual *Rediviva* species usually collect oil from more than one *Diascia* host species. Thus, leg length might experience diffuse selection pressures from the spurs of several *Diascia* species in the local community rather than just from an individual host species, resulting in complex dynamics and potentially accelerated evolution of leg length observed in our study.

Interestingly, we also failed to detect a close correlation between leg length and ecological specialisation in oil host usage of *Rediviva* species. Leg elongation does not seem to restrict the oil host spectrum, a finding also revealed by Borrell (2005), who suggested that longer feeding appendage give pollinators the ability to access a wider range of resources. These findings suggest that, although leg elongation results in phenotypic specialisation, it seems not to be linked to ecological specialisation.

4.4. *Rediviva* distribution patterns and the geology of the Greater Cape Floristic region (GCFR)

According to our reconstruction of ancestral biogeography, the ancestor of *Rediviva* and *Redivivoides* probably arose in the winter rainfall area. Since no extant taxon occurs in both summer and winter rainfall areas, and the few species found in the summer rainfall area are scattered through the phylogenetic tree, several dispersal and vicariance events might have occurred later on in the evolution of the genus. Two of these vicariance events were probably experienced by the ancestors of *R. saetigera* and *R. colorata* and their respective sister groups at approximately 17 MYA, around the time of origin of the Succulent Karoo vegetation region (Verboom et al., 2009, see below), in the middle Miocene, a time of relative climate stability with elevated temperatures and high humidity and rainfall (Sciscio et al., 2013). One other vicariance event was inferred for the ancestor of the E clade to *R. gigas* and the clades B, C and D which happened c.a. 6 MYA, in the late Miocene/early Pliocene, when the climate in the GCFR rapidly cooled down, became more arid and shifted to a winter rainfall regime due to the development of the westerly wind system and strengthening of the cold Benguela current (Cowling et al., 2009; Neumann and Bamford, 2015). The Post-African I and II erosion cycles in the early and late Miocene and climatic stability are assumed to have directly stimulated the diversification of the GCFR vegetation (Cowling et al., 2009; Neumann and Bamford, 2015; Schnitzler et al., 2011), which might have also strongly impacted the local fauna and might have caused several vicariance events.

4.5. Pollinators and plant diversity in the GCFR

Although the historical and recent macroevolutionary processes causing the great diversification and biodiversity of the GCFR are still under debate and might result from a combination of abiotic and biotic factors (Schnitzler et al., 2011; Verboom et al., 2009), pollinators are very likely to be important for driving plant speciation on a micro-evolutionary scale (Johnson, 2010). At the same time, host plants might shape the evolution of their pollinators.

Interestingly, the GCFR is the only place worldwide where a plant and bee diversity hotspot coincide (Kuhlmann, 2009), with the Succulent Karoo showing the greatest species richness and highest levels of endemism (Kuhlmann, 2009). The origin of the Succulent Karoo has been estimated to be around 17.5 MYA and was probably followed by a rapid radiation of the local flora with a peak at the transition of the Miocene to the Pliocene (Cowling et al., 2009; Verboom et al., 2009). The origin of the succulent vegetation fits the timeframe of the majority of *Rediviva* speciation events (see above), supporting the correlated evolution of local pollinator fauna and their host plants. *Rediviva*'s preferred host plant, *Diascia*, might have co-speciated with *Rediviva* bees as the plant genus likely split from its sister clade *Nemesia* (Datson et al., 2008: 32–26 MYA or Renner and Schaefer, 2010: 15 (24 − 4) MYA) at approximately the same time we date the origin of the common ancestor of *Rediviva* and *Redivivoides*, i.e. 29 MYA.

Climate seems to be highly important for generating the diversity of plants and pollinators, not only in the past but also in the present. The special precipitation regime in the winter rainfall area might lead to a reduction of daily activity and small foraging ranges in bees (Kuhlmann et al., 2012), potentially resulting in reduced gene flow among populations in both bees and plants and possibly leading to speciation (Linder et al., 2010; Van der Niet et al., 2014). High speciation rates in the GCFR (Linder et al., 2010; Verboom et al., 2009) are exemplified not only by the great diversity of the local flora (Myers et al., 2000; Schnitzler et al., 2011) but also by the existence of multiple specialised plant-pollinator systems (Johnson, 2010) with reciprocal adaptations in flower visitor and plant, such as long-legged *Rediviva* species and their long-spurred *Diascia* hosts, long-tongued insects (hawkmoths, nemestrinid and tabanid flies) or long-billed birds and their long-tubed host-plants (Alexandersson and Johnson, 2002; Geerts and Pauw, 2009; Goldblatt and Manning, 2000; Pauw et al., 2009) or plants pollinated by rodents (Johnson et al., 2001; Kleizen et al., 2008).

Overall, our study suggests that foreleg length seems to be a highly flexible and fast evolving trait that arose at least two times within *Rediviva.* Moreover, we established five major clades within *Rediviva* and, in accordance with previous research, failed to support the monophyly of the genus *Rediviva* because *Redivivoides* consistently nested within *Rediviva*. Thus, a taxonomic revision of *Rediviva*/*Redivivoides* is required. Our *Rediviva* phylogeny also represents the basis for co-phylogenetic studies between *Rediviva* and its main host *Diascia*, which will help to address the question of co-evolutionary interactions in this fascinating plant-pollinator system.

Acknowledgements

We thank Martin Husemann for discussion and advice, Savel Daniels for suggesting the variant of CO1 sequenced here, as well as Antonella Soro, Robin Moritz and Michael Lattorf for helpful discussion. The Northern Cape Nature Conservation Service and the Western Cape Nature Conservation Board (CapeNature) kindly gave their permission to collect bees in the winter rainfall area of South Africa. This work was partially supported by U.S. National Science Foundation grants to Bryan Danforth (DEB-0814544, DEB-0742998). Sequencing was also partially funded by the German Centre for Integrative Biodiversity Research (iDiv). Belinda Kahnt was supported by a stipend of the Studienstiftung des Deutschen Volkes.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.07.025.

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