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Molecular phylogenetic analysis of archival tissue reveals the origin of a disjunct southern African–Palearctic weevil radiation

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ABSTRACT

Aim We test three alternative hypotheses for the disjunct Mediterranean–southern African distribution of endophagous weevils within the genera *Rhinusa* and *Gymnetron* (Coleoptera: Curculionidae): (1) a Palaearctic origin with dispersal to southern Africa; (2) a southern African origin with dispersal to the Palaearctic; and (3) a widespread ancestral distribution fragmented by vicariance. Divergence times are estimated to provide an approximate temporal framework for the evolution of the group and to evaluate potential palaeogeographical scenarios.

Location Southern Africa, the Mediterranean region, the Palaearctic and eastern Africa.

Methods Freshly collected and dry, pinned samples of weevils were used as a source of DNA. Prior genetic information was used to identify short phylogenetically informative amplicons within the 16S ribosomal RNA gene (*16S*). Phylogenetic reconstructions using Bayesian and maximum likelihood analyses of mitochondrial and nuclear DNA sequence data and molecular dating techniques were used to infer the biogeographical history of *Rhinusa* and *Gymnetron* species. A statistical approach to dispersal–vicariance analysis (S-DIVA) was used to further assess biogeographical hypotheses.

Results Successful polymerase chain reaction amplification of targeted short *16S* DNA sequences (150 bp) from dry, pinned specimens provided for increased species sampling of *Rhinusa* and *Gymnetron* by 230%, greatly expanding species representation from southern Africa. Phylogenetic reconstructions and S-DIVA analyses support a southern African origin for *Rhinusa* and *Gymnetron* species. Divergence time estimates suggest southern African and Palaearctic lineages diverged approximately 11.6–7.4 Ma.

Main conclusions *Rhinusa* and *Gymnetron* represent a complex of lineages with a shared evolutionary history of range expansions from southern Africa into the Palaearctic. Our results support a late Miocene vicariance scenario, most likely as a result of repeated

desertification. The use of prior genetic information to identify short phylogenetically informative amplicons offers a useful approach for molecular phylogenetic analyses incorporating archival material.

Keywords

Archival tissue, Curculionidae, disjunct distribution, dispersal, Palaearctic, phylogeny, southern Africa, vicariance, weevil.

INTRODUCTION

Examples of Mediterranean–southern African disjunct distributions are perhaps mostly known in plants, given the floristic affinities shared by these two regions, which comprise two of the five mediterranean-type floras of the world (Cowling *et al.*, 1996). Previous studies have proposed long-distance dispersal as the major cause of this disjunction (Thorne, 1972; Raven, 1973) and, with some exceptions (e.g. McGuire & Kron, 2005), the most widely accepted hypothesis is a southern African origin with dispersal to the north through an East African corridor (Caujapé-Castells *et al.*, 2001; Coleman *et al.*, 2003; Calviño *et al.*, 2006; del Hoyo *et al.*, 2009). Interestingly, there are very few studies on plant-feeding insects that exhibit similar disjunct distributions, perhaps tracking the ancestral distribution of their host plants (but see Mey, 2006, and Kirk-Spriggs & McGregor, 2009, for examples in Lepidoptera and Diptera, respectively). Within Coleoptera, proposed explanations for south African–Mediterranean disjunct distributions favour a vicariance scenario (Biondi & D’Alessandro, 2008; Bologna *et al.*, 2008), acknowledging the possible ecological connections between the southern African and Mediterranean regions in the past via ‘arid corridors’, as originally described by Balinsky (1962), which are thought to have appeared in eastern Africa since the end of the Miocene (Verdcourt, 1969; Goldblatt, 1978; Jürgens, 1997).

To further investigate disjunct Mediterranean–southern African insect distributions, we focus attention on weevil species of the closely related genera *Rhinusa* Stephens, 1829 and *Gymnetron* Schoenherr, 1825, within the tribe Mecinini (Curculionidae: Curculioninae). Adults of this species-rich group range in size from 2 to 5 mm, with representatives in both the Palearctic and Afrotropical regions. Species of both genera are endophagous parasites whose larvae feed and develop within tissues of plants in the families Scrophulariaceae and Plantaginaceae as recently circumscribed (Olmstead *et al.*, 2001; Albach *et al.*, 2005). The

genus *Rhinusa* comprises approximately 40 species following a Palaearctic distribution (Caldara, 2001), feeding on species within the plant families Scrophulariaceae (*Verbascum* and *Scrophularia*) and Plantaginaceae (*Linaria*, *Kickxia*, *Chaenorhinum*, *Antirrhinum* and *Misopates*) (Caldara *et al.*, 2010). *Gymnetron* includes approximately 30 species with a Palaearctic distribution and approximately 60 species from the Afrotropical region, of which 55 are known mainly from South Africa and considered to be endemic to this area (Caldara, 2003). All Palaearctic species use plant species from the genus *Veronica* (Plantaginaceae) as host plants, whereas representatives from the Afrotropical region use species within the family Scrophulariaceae (*Hebenstretia*, *Sutera*, *Selago*, *Buddleja*, *Diascia*, *Nemesia* and *Hemimeris*) and the genus *Anastrabe* in the family Stilbaceae (Caldara *et al.*, 2008). Both genera are taxonomically challenging, with very few morphologically informative characters (Caldara *et al.*, 2010) and, until recently, *Rhinusa* was considered a subgenus within *Gymnetron* (Caldara, 2001). In a recent revision of the relationships between Mediterranean and southern African species within the subfamily Curculioninae, Caldara *et al.* (2008) concluded that species from the Palaearctic and Afrotropical regions appear to be very closely related with only a few subtle morphological differences. However, they recognize that it is not currently possible to distinguish between hypotheses of dispersal or vicariance, nor whether these genera may have originated in southern Africa or in the Mediterranean.

To test among competing biogeographical hypotheses for disjunct Mediterranean and southern African insect distributions, we use sequence data from two mitochondrial and three nuclear genomic regions to reconstruct phylogenetic relationships of representatives within *Rhinusa* and *Gymnetron*. These taxa represent an ideal example of a focal group that requires a molecular phylogenetic approach to understand the origin of its species diversity; access to many species is, however, limited to archival material, particularly for southern African taxa. Therefore, we maximize our taxonomic and geographical coverage by

exploiting the use of archival specimens from a pinned, dried collection using a non-destructive approach. We use genetic data sampled from non-archival material within our focal group as prior information for the design of primers to amplify short phylogenetically informative amplicons (SPIAs; < 100 bp) of the mitochondrial 16S ribosomal RNA gene, for the incorporation of archival material in molecular phylogenetic analyses. We aim to maximize the number of phylogenetically informative sites, while minimizing amplicon length to account for the limitations in amplifying degraded DNA from museum specimens.

Thus, using both archival specimens and freshly collected samples we test three alternative hypotheses for the disjunct Mediterranean–southern African distribution of *Gymnetron* species: (1) a Palaearctic origin with dispersal to southern Africa; (2) a southern African origin with dispersal to the Palaearctic; and (3) a widespread ancestral distribution fragmented by vicariance. Divergence times are estimated to provide an approximate temporal framework for the evolution of the group and evaluate potential palaeogeographical scenarios.

MATERIALS AND METHODS

Taxon sampling

Our sampling strategy was to obtain broad geographical and taxonomic coverage of representatives within *Gymnetron* and *Rhinusa*, using freshly collected samples where possible, and augmenting our sampling for material that was harder to obtain using archival specimens (see Appendix S1 in Supporting Information). Sixty-seven specimens were collected in the field, placed in 96–100% ethanol, and stored at 4 °C until DNA extraction. Forty-two dry, pinned specimens were used from the personal entomological collection of R. Caldara for DNA extraction from a single leg (see below). Thirty-two species of *Rhinusa* were sampled (18 field collected and 14 archival), representing approximately 80% of

recognized species, including representatives from the three main taxonomic groups proposed by Caldara *et al.* (2010). Twenty-five species of *Gymnetron* were sampled (6 field-collected and 20 archival; one species – *G. villosulum* – was sampled both fresh and archivally), representing 8 of the 13 Afrotropical groups proposed by Caldara (2003) and 11 species from the Palearctic region. One specimen of the genus *Cleopomiarus* was also included, which is the only genus within the tribe Mecinini apart from *Gymnetron* to have representatives in both the Palearctic and Afrotropical regions (Caldara *et al.*, 2008).

DNA extraction

Field-sampled weevils were punctured through the abdomen and total genomic DNA was extracted from each individual using the DNeasy extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. After DNA extraction, insects were placed again in 96% ethanol and kept at 4 °C, before subsequent preparation as voucher specimens within the collection of R.C. When using archival specimens, destruction of the sample for DNA extraction has always been a concern, especially for small specimens (Thomas, 1994; Whitfield & Cameron, 1994), and for arthropods, several methods have been proposed to minimize damage (Gilbert *et al.*, 2007; Rowley *et al.*, 2007). We use a non-destructive approach using a single leg for DNA extraction as suggested by Gilbert *et al.* (2007). Dry, pinned insects were first left overnight in a humid chamber in order to reduce exoskeleton brittleness and allow manipulation of the appendages. A single posterior leg was then removed under a dissecting microscope and placed in 200 µL of DNeasy extraction buffer for overnight incubation at 56 °C, followed by DNA isolation as for non-archival samples. DNA-extracted appendages were recovered and remounted with their corresponding specimen on a new entomological card (Appendix S2: Fig. S1).

PCR amplification and sequencing reactions

Two mitochondrial [cytochrome *c* oxidase subunit II (*COII*) and 16S ribosomal RNA (*16S*)] and three nuclear gene fragments [elongation factor-1 α (*EF-1 α*), arginine kinase (*AK*) and 18S ribosomal RNA (*18S*)] were used. The primers used for each gene are described in Appendix S3. For all loci, polymerase chain reactions (PCR) were performed with BioTaq DNA polymerase (Bioline, London, UK) with 1 $\times$ NH₄ buffer, 3.5–5.0 mM MgCl₂, 0.2 mM each dNTP, 0.2–0.4 μ M each primer, 0.5 U of Taq polymerase and 1–5 μ L of DNA template in a 25- μ L final volume. PCR cycles were carried out using the following thermal profile for the *COII* and *AK* gene fragments: 95 °C for 3 min, 33 and 37 cycles respectively at 95 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 3 min. For *EF-1 α* , *16S* and *18S* genes, two different touchdown profiles (Don *et al.*, 1991) were used. For *EF-1 α* and *16S*, the programme comprised 94 °C for 1 min 30 s, 10 cycles at 94 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min, decreasing the annealing temperature by 1 °C every cycle, then 35 cycles at 94 °C for 45 s, 48 °C for 1 min, 72 °C for 1 min, and 72 °C for 3 min as a final extension. For the *18S* fragment, the programme comprised 95 °C for 3 min, 8 cycles at 94 °C, 54 °C and 72 °C for 1 min at each temperature, decreasing the annealing temperature by 1 °C every cycle, then 28 cycles at 94 °C, 46 °C and 72 °C for 1 min at each temperature and a final extension at 72 °C for 2 min. Sequences were generated with a PerkinElmer ABI3700 automated sequencer, using the BigDye terminator reaction protocol (v3.1 PerkinElmer, Cheshire, UK) in a 10- μ L final volume. For the *COII*, *16S*, *AK* and *18S* gene fragments, sequences were obtained with the forward primer only, except in few cases which required sequencing in both directions because of low-quality chromatograms. All of the *EF-1 α* sequences were obtained with forward and reverse primers because of the presence of an intron region with indels.

SPIAs primer design, PCR amplification and sequencing

Based on the alignment of gene partitions from non-archival samples, we identified two adjacent variable regions of 95 bp and 55 bp within the mitochondrial *16S* gene, flanked by comparatively conserved motifs. We designed primers spanning each of these amplicons, also including representative sequences from GenBank within the family Curculionidae to maximize the broad taxonomic utility of these primers, including the following genera: *Anthonomus*, *Brachonyx*, *Curculio*, *Eutoxus* and *Tychius*. We also included *Tribolium* from the family Tenebrionidae. For one of the *16S* SPIAs, the forward PCR primer included an M13 adaptor oligonucleotide (Appendix S3), and the adaptor was used as a sequencing primer to improve sequence read quality immediately after the 3' end of the primer. The two *16S* amplicons were PCR amplified with the following conditions: 95 °C for 5 min; 40 cycles at 95 °C; 45 °C and 72 °C for 20 s; and a final extension at 72 °C for 5 min. To control for contamination, full precautions were taken; these included the use of a PCR hood workstation with a 15-minute UV-light sterilization step, dedicated PCR pipettes, filtered tips, and the inclusion of multiple negative controls. All short *16S* amplicons were sequenced in both directions, and in some cases resequenced from an independent PCR reaction to corroborate accuracy.

Phylogenetic analyses

All sequences were automatically aligned using the CLUSTALW algorithm, as implemented in BIOEDIT 7.0.9 (Hall, 1999), with further manual alignment. For *EF-1 α* , forward and reverse sequences were first assembled as contigs, and the intron region removed due to the inability to align it unambiguously. Sequence properties for each individual gene partition were assessed using MEGA 4.0.1 (Tamura *et al.*, 2007). Phylogenetic analyses were performed for individual partitions, and for a concatenated alignment of nuclear and

mitochondrial sequences. For the latter, analyses were performed using two data sets. The first data set consisted of ingroup taxa with complete sampling of the five gene partitions. The second data set consisted of the first data set with the addition of archival specimens sampled for the SPIAs. Bayesian analyses were performed with the parallel version of MRBAYES 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using the substitution models selected by JMODELTEST (Posada, 2008) for each partition, with priors set to the default values as recommended by Ronquist *et al.* (2005). Settings for each gene partition in the individual analyses were as follows: two simultaneous runs (each with two chains) of the Markov chain Monte Carlo (MCMC) for five million generations, with a sampling frequency of 100 generations, a heating parameter value of 0.02–0.05 (decreased from the default value 0.2 to improve swapping of states between the heated and cold chains) and a relative burn-in of 25%. Summaries from the stationary distribution of the sampled parameter values and sampled trees were obtained, as well as a majority-rule consensus tree with posterior probabilities for each bipartition. For concatenated sequence analyses (five gene partitions) with and without archival specimens, we also used two independent runs but with four Markov chains each, to optimize convergence for large data sets, as suggested by Ronquist *et al.* (2005), and 20 million generations, sampling every 1000 generations. To determine stationarity of the MCMC chains, variation in log-likelihood scores was examined graphically using the program TRACER 1.5 (Rambaut & Drummond, 2007); the final standard deviation of split frequencies was ≤ 0.01 in all analyses. Maximum likelihood (ML) analyses were performed with the parallel version of RAXML 7.0.4 (Stamatakis, 2006) for the data set of five gene partitions with and without archival specimens sampled for the SPIAs. A partitioned model was used where each genetic marker was assigned a separate GTR+I+G model. One thousand heuristic searches were executed using the default settings to find the ML tree. Branch support was estimated from 1000 replicates using the standard bootstrap

procedure as implemented in RAXML (Stamatakis *et al.*, 2008). Both Bayesian and ML analyses were conducted on the High Performance Computing Cluster at the University of East Anglia.

Assessing the phylogenetic placement of archival material sampled for SPIAs

Phylogenetic placement of the archival taxa sampled for SPIAs was assessed with two approaches. First, we performed a Bayesian analysis of non-archival specimens with the concatenated alignment of five gene partitions. Nucleotide data for selected taxa representing a range of divergences from sister lineages were reduced or ‘trimmed’ to the SPIA partitions. We then compared the phylogenetic placement of these character-reduced taxa within the phylogeny with their placement in analyses using the full set of characters. As a second approach, Bayes factors were used to compare the likelihoods of models with enforced monophyly at key nodes of interest including archival specimens sampled for the SPIAs, against models without such constraints.

Estimation of divergence times

In the absence of geological or fossil calibration points to estimate divergence times, we take a Bayesian approach as implemented in BEAST 1.4.8 (Drummond & Rambaut, 2007) using a relaxed molecular clock to estimate the age of the most recent common ancestor (MRCA) of nodes of interest representing divergences between southern African and Palearctic lineages. We applied a mean pancrustacean *COII* rate estimate of 3.05% pairwise divergence per million years, based on previous work showing the mean substitution rate of Coleoptera to approximate the mean rate across the Pancrustacea (Cicconardi *et al.*, 2010). The estimated age of the root of the *COII* tree was then used as prior information for rate calibration of the SPIAs. The validity of applying the *COII* mean substitution rate to the *16S*

gene was assessed by comparison of the uncorrected pairwise distances of both genes which are closely correlated ($r = 0.74$) (see Appendix S2: Fig. S2).

Reconstruction of ancestral ranges

To further evaluate the biogeographical patterns observed in the phylogeny and infer ancestral ranges, we used statistical dispersal–vicariance analysis as implemented in the software S-DIVA (Yu *et al.*, 2010). This program incorporates the methods of Nylander *et al.* (2008) and Harris & Xiang (2009) to account for phylogenetic uncertainty – and uncertainty in dispersal–vicariance analysis (DIVA) optimization – using a nonparametric empirical Bayesian approach. Statistical support for ancestral range reconstructions is determined by averaging the frequencies of an ancestral range at a node over a set of trees (Yu *et al.*, 2010). We used the 20,000 trees sampled from the Bayesian analysis of the data set of five gene partitions including archival specimens with 5000 trees discarded as burn-in. The number of maximum areas was set to two, reflecting the ingroup’s number of defined areas of distribution – (A) southern Africa and (B) Palaearctic.

RESULTS

The five gene fragments were amplified and sequenced for 56 of the 67 field-collected samples (see Appendix S1 for GenBank accession numbers). The 10 samples of *G. rotundicolle* and the sample of *G. rostellum* consistently failed to amplify for any gene fragment, and were subsequently found to have been stored in denatured ethanol. They were consequently amplified using the same protocol applied to archival samples. Across the 56 individuals sequenced for the five gene partitions, only five samples present some missing sequence data due to poor read quality in some regions of the chromatograms. Sequence variation within each gene partition is detailed in Table 1. The combined data of five gene

partitions contained 3943 nucleotides, of which 885 sites were variable and 19% were parsimony-informative. The full DNA sequence data matrix including SPIA sequences is available in TreeBase (<http://treebase.org/>, accession number S13611).

SPIA primer design, PCR amplification and sequencing

Successful PCR amplification was achieved for both SPIAs for all 42 archival samples and the 11 samples in denatured ethanol. Five archival samples yielded comparatively weak PCR products, and did not generate readable sequence chromatograms. The remaining 37 samples yielded readable sequence chromatograms for both SPIAs (Appendix S1), increasing our species sampling with the addition of 11 and 17 taxa from *Rhinusa* and *Gymnetron*, respectively, and greatly expanding species representation from southern Africa.

Phylogenetic analyses and assignment of archival specimens

Analyses of individual gene partitions essentially recovered the same tree topology, but with different degrees of phylogenetic resolution. Bayesian and ML analyses of the data set of five gene partitions, without archival samples, recovered the same robustly supported phylogeny (Fig. 1), revealing neither *Rhinusa* nor *Gymnetron* to be monophyletic, but with phylogenetic relationships among *Rhinusa* species broadly in agreement with the taxonomy of *Rhinusa* based on morphological characters. The same phylogenetic relationships among non-archival samples were derived from Bayesian and ML analyses when the *16S* SPIAs were included within the alignment of the five gene partitions (Fig. 2), with the single exception of *G. melanarium* being placed basally within clade G in the Bayesian analysis. Bayesian and ML analyses with the inclusion of the *16S* SPIAs also resulted in the identical phylogenetic placements of the archival specimens (Fig. 2), with the single exception of clade I being placed as the sister group to clade L in the ML analysis.

Despite the limited amount of nucleotide data (150 bp), relatively high posterior probability (PP) and/or bootstrap support (BS) values were observed for the placement of some archival specimens. Two southern African taxa, *Gymnetron perrinae* and *G. bisignatum*, are united as sister taxa with PP = 1 and BS = 93%. *Rhinusa exigua* is placed as the sister lineage to the clade of *R. antirrhini*/*R. florum* with PP = 0.85 and BS = 78%. *Rhinusa brisouti* is placed as the sister lineage to *R. linariae* with PP = 0.90 and BS = 75%. *Rhinusa moroderi* is placed as the sister lineage to *R. tetra* with PP = 0.98 and BS = 85%. *Rhinusa comosa* is placed as the sister lineage to *R. verbasci* with PP = 1 and BS = 98%. *Gymnetron minimum* and *G. paucillum* are united as sister species with PP = 1 and BS = 99%. Finally, *G. aequale* and *G. veronicae* are united as sister taxa with PP = 1 and BS = 100%. Twenty-four archival specimens are placed within clades A, B, E, F and G (Fig. 2), which were also inferred by the analysis of the five-gene data set without archival specimens (Fig. 1). The remaining 13 samples, plus the 10 samples of *G. rotundicolle* and the sample of *G. rostellum*, were placed outside the lineages and clades defined in Fig. 1.

To examine how placements might be influenced by limited nucleotide data, a Bayesian analysis of the concatenated alignment of the five genes without archival samples was performed with the sequences of *R. linariae*, *R. vestita* and *G. piceum* trimmed to represent the 16S SPIAs. In the case of *R. linariae* and *R. vestita*, representing moderate and intermediate divergences, respectively, from their sister lineages, they were both assigned to their correct clades, but with some slight topological changes. A single branch arrangement places *R. linariae* as a sister lineage to *R. griseohirta* within clade A (see Appendix S2: Fig. S3). Two branch arrangements result in a more derived phylogenetic position for *R. vestita* within clade B. In the more extreme case of divergence from a sister lineage, the phylogenetic placement of *G. piceum* was substantially altered (Fig. S3). These results indicate that while approximate phylogenetic placement can be achieved with limited SPIA

data, exact placements might not be reliably inferred. Thus, our phylogenetic results clearly support deep genetic divergences across southern African taxa, although the specific relationships of southern African lineages to Palaearctic lineages and the basal divergences of several southern African lineages require further assessment. To achieve this, we carried out Bayes-factor tests to compare the harmonic means of likelihood values of (1) models with enforced monophyly for each of nodes I–IV (Fig. 2) that represent the MRCAs of southern African and Palaearctic lineages, and (2) a model enforcing the monophyly of clades A–L, excluding all remaining southern African species, against models without the constraint of monophyly. Applying the guidelines of Kass & Raftery (1995), we obtained very strong support for the monophyly of nodes II–IV, and the clade comprising groups A–L ($\ln \text{Bayes factor} > 10$), while only moderate support was obtained for the monophyly of node I ($2 < \ln \text{Bayes factor} < 10$).

Divergence time estimates

BEAST analyses calibrated with the evolutionary rate for the mtDNA *COII* gene generated a mean estimate of 26.7 million years ago (Ma) for the MRCA of *Gymnetron* and *Rhinusa*, with a 95% highest posterior density (HPD) interval of 21.2–32.5 million years (Ma). This age estimate was used as prior information to calibrate the corresponding node of the tree containing both archival and non-archival samples (Fig. 2), to estimate the approximate divergence times of southern African *Gymnetron* lineages from Palaearctic *Gymnetron* and *Rhinusa* lineages with *16S* SPIA sequence data. We imposed a normal distribution for the root age with a mean of 26.7 Ma and a standard deviation of 7 Myr, allowing a 95% probability distribution for sampling of 15–35 Ma, an interval that includes the 95% HPD estimated with mtDNA *COII* sequence data. Prior information for the *16S* SPIA substitution rate was also incorporated. We estimated a range of *16S* SPIA rates by fixing the MRCA of

Gymnetron and *Rhinusa* to be (1) 21.2 Ma, representing the lower 2.5% posterior probability value estimated from mtDNA *COII* data, and (2) 32.5 Ma, representing the upper 2.5% posterior probability value. Based on the resulting mean rate estimates from these calibrations of 0.0047 and 0.018 substitutions per site per million years, we applied a normal distribution for the SPIA mutation rate with a mean of 0.01 and a standard deviation of 0.005, thus incorporating our estimated range within the 95% HPD interval. Normal distributions were chosen as prior parametric distributions because they allow bidirectional uncertainty in the calibration estimates (Ho & Phillips, 2009). Ages for four nodes corresponding to divergences between southern African and Palaeartic lineages were estimated (Fig. 2, Table 2), yielding broad HPDs, reflecting the broad prior root age and substitution-rate distributions that we allowed the analysis to sample from. However, the mean age estimates for all four divergence events range between 11.6 and 7.4 Ma, suggesting a late Miocene divergence between Palaeartic and southern African lineages.

Reconstruction of ancestral ranges

The relative probabilities of alternative ancestral ranges mapped onto the two most basal nodes of the phylogenetic tree that included the archival specimens suggest a southern African origin for *Rhinusa* and *Gymnetron*, with values higher than 60% (Fig. 2). Subsequent vicariant events across ancestral ranges that extended to include both southern Africa and the Palaeartic were inferred for nodes representing the MRCAs of southern African and Palaeartic lineages (nodes I–IV; Fig. 2).

DISCUSSION

Phylogenetic analyses and assignment of archival specimens

Analyses of *Rhinusa* and *Gymnetron* species sampled for all five gene partitions resulted in a robust phylogeny, recovering four monophyletic groups largely in agreement with a proposed taxonomy based on adult morphological characters (Caldara *et al.*, 2010). The single major difference from conventional taxonomic classification is that *Gymnetron* and *Rhinusa* are not reciprocally monophyletic. When the *16S* SPIAs from archival specimens were included in the data matrix, Bayesian and ML analyses recovered essentially the same tree topology, with most archival samples falling within clades defined by the complete matrix of five genes, largely in agreement with prior taxonomic expectations (Caldara, 2003, 2008; Caldara *et al.*, 2010). High support values for the placement of several archival specimens, obtained in both Bayesian and ML analyses, suggest that phylogenetic placement can in some cases be achieved with a high degree of confidence using SPIAs. This is consistent with previous findings that show there is no relationship between character completeness of a taxon, and the level of support for its placement within a phylogeny (Wiens *et al.*, 2005). However, for more divergent archival lineages within a particular clade, phylogenetic placement may be less reliably inferred, as revealed by our Bayesian analyses with sequences for selected taxa reduced to the 150 nucleotides of the *16S* SPIAs. Thus, for nodes of relevance to our biogeographical hypotheses that lack high support from both Bayesian posterior probabilities and ML bootstrap values, we have applied Bayes-factor analyses to test hypotheses of monophyly, and applied statistical dispersal–vicariance analyses, allowing us to take phylogenetic uncertainty into account.

Although several authors have suggested that including taxa with a high proportion of missing characters is potentially problematic for phylogenetic reconstruction (Huelsenbeck, 1991; Wilkinson, 1995; Kearney, 2002; Hartmann & Vision, 2008), our results are largely in agreement with evidence suggesting that as long as taxa with missing characters bear sufficient phylogenetically informative data, and the overall number of

characters included in the analysis is not unusually low (≤ 100), highly incomplete taxa can be accurately placed within a phylogeny (Wiens & Morrill, 2011; and references therein). It has previously been suggested that parametric methods, such as ML and Bayesian analyses, perform well in placing taxa with missing characters onto individual branches with high support values by extrapolating topological and branch length inferences from one group of characters to another (Philippe *et al.*, 2004; Fulton & Strobeck, 2006; Wiens & Moen, 2008; Wolsan & Sato, 2010). Using a sequence data matrix of 3943 bp as a ‘scaffold’ on which the missing-character taxa could be placed (Wiens *et al.*, 2005; Wiens, 2006), we have shown that the geographical and taxonomic scope of phylogenetic studies can be augmented with archival specimens by the targeted amplification of short phylogenetically informative DNA regions.

Historical biogeography of *Rhinusa* and *Gymnetron*

Southern African taxa are characterized by deep genetic divergences, several lineages of which form monophyletic groups with Palearctic *Rhinusa* and *Gymnetron* lineages. Bayes-factor analyses support a phylogenetic topology with basal lineages of southern African origin. Following the reasoning of Bremer (1992) and Hausdorf (1998), plesiomorphic areas in a cladogram are more likely to be part of the ancestral range than apomorphic areas; likewise, areas represented on more than one branch have a higher probability of being part of the ancestral area than areas less represented. Thus, the basal placement of three southern African lineages within the phylogeny (*G. gossypinus*, *G. bisignatum* + *G. perrinae*, and *G. buddleiae*) supports southern Africa as the ancestral area from where extant *Gymnetron* and *Rhinusa* species diversity is derived. Congruent with this hypothesis, results from the S-DIVA analysis show higher relative probabilities for southern Africa as the ancestral range at the two most basal nodes in the phylogeny (Fig. 2).

One of the more likely opportunities for large-scale range expansions from Africa into the Palaearctic, or the Palaearctic into Africa, probably occurred during the early–mid Miocene boundary (*c.* 17 Ma), when a land connection formed between Europe and Africa after the closure of the Tethys Sea (Levyns, 1964; McGuire & Kron, 2005). Several authors have suggested the existence of geographical and ecological routes connecting southern and eastern Africa with the Mediterranean region in the past that would have facilitated range expansions of plants and animals between the Palaearctic and Afrotropical regions. Evidence supporting the presence of an ‘arid corridor’ connecting southern, eastern and north-eastern African areas prior to the end of the Miocene has been documented for many plant groups (Verdcourt, 1969; Goldblatt, 1978; Jürgens, 1997; Caujapé-Castells *et al.*, 2001; Coleman *et al.*, 2003; Thiv *et al.*, 2011) and animals (Balinsky, 1962; La Greca, 1990; Freitag & Robinson, 1993; Herron *et al.*, 2005) exhibiting disjunct distributions. Severance of this corridor has been linked to late-Miocene uplift and associated rifting in Africa and major climatic changes during the late Miocene (Chorowicz, 2005; Schuster *et al.*, 2006). Both our age estimates and S-DIVA analyses are consistent with this scenario. Although our analyses yielded broad HPD intervals, reflecting the broad prior root age and substitution rate distributions employed, mean estimated divergence times between Palaearctic and southern African lineages (nodes I–IV; Fig. 2) suggest that divergences most probably occurred within the late Miocene (*c.* 11.6–7.4 Ma). S-DIVA analysis inferred vicariant events and an ancestral range including both southern Africa and the Palaearctic regions at nodes representing the MRCA of southern African and Palaearctic lineages (nodes I–IV; Fig. 2). Further support for the east African arid corridor hypothesis comes from the presence of several *Gymnetron* species in eastern Africa (Caldara *et al.*, 2008), suggesting more northerly African relicts of an ancestral range once stretching north from southern Africa to East Africa. The land connection between the African and Asian plates would have facilitated range expansion

further north into the Mediterranean and European regions, with new vegetation zones representing the opportunity to exploit new niches. Climate-mediated vicariant events would have acted to fragment a once-continuous range as conditions became progressively drier after the mid-Miocene (Axelrod & Raven, 1978), with periods of greater aridity, particularly for the eastern region of Africa, in the late Miocene (*c.* 6 Ma), the Pliocene (*c.* 3 Ma), and near the Pliocene–Pleistocene boundary (< 2 Ma) (deMenocal, 1995; Bobe, 2006). The importance of these climatic changes as vicariant events has been demonstrated for different groups of animals (Leakey *et al.*, 1996; Douady *et al.*, 2003) and plants (del Hoyo *et al.*, 2009; Thiv *et al.*, 2010), including examples of mid-to-late Miocene radiations in the genera *Nemesia* (Datson *et al.*, 2008) and *Linaria* (Fernández-Mazuecos & Vargas, 2011), two host plant genera utilized by *Gymnetron* and *Rhinusa*, respectively.

Within Coleoptera, our results are in accordance with previously proposed vicariance scenarios for Mediterranean–southern African disjunct distributions of beetles in the families Meloidae (Bologna *et al.*, 2008) and Nitidulidae (Audisio *et al.*, 2008). For both these groups, repeated desertification phenomena since the Miocene and through the Pliocene and Pleistocene are suggested to have fragmented probable ancestral distributions extending between European–Mediterranean and eastern/southern African areas. It should be noted that our divergence-date estimates may overestimate the timing of divergence between southern African and Palaearctic lineages, due to incomplete species sampling and species extinctions. Additionally, our analyses have incorporated broad parametric prior distributions to account for uncertainty both in tree root age, and in molecular evolutionary rate, yielding wide HPD intervals for our estimated divergence times. However, the broad consistency across our four mean age estimates, together with ancestral range reconstruction, argue for a southern African origin of species diversity within *Gymnetron* and *Rhinusa*, with subsequent range expansion into the Palaearctic followed by late-Miocene vicariance and divergence

between southern African *Gymnetron* lineages and lineages of *Gymnetron* and *Rhinusa* in the Palaearctic.

CONCLUSIONS

Rhinusa and *Gymnetron* are not monophyletic with respect to each other, and present multiple lineages that are disjunct between southern Africa and the Palaearctic. We were able to test biogeographical hypotheses for the origin of their southern African–Palaearctic disjunct distribution by incorporating many species only available as archival material, using prior genetic information to identify short phylogenetically informative amplicons for their approximate phylogenetic placement. Molecular phylogenetic data provide support for a southern African origin, with the earliest divergence within the group estimated to date back to approximately 27 Ma, and subsequent range expansion into the Palaearctic. The timing of the northward range expansion of the group into the Palaearctic is not discernible from the tree topology, but would have been facilitated by a Miocene ‘arid corridor’, suggested to have connected southern, eastern and north-eastern African areas. Molecular data permit estimation of the approximate timings of four vicariant events between Palaearctic and southern African lineages, which are consistent with an inferred late-Miocene disruption of the arid corridor as a consequence of geological and climatic changes within this period.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Field-sampled and archival specimens used in this study with GenBank accession numbers except for samples whose DNA was amplified for the short *16S* amplicons only.

Appendix S2 Supplementary figures. Leg of *Gymnetron villosulum* remounted on an entomological card after DNA extraction (Fig. S1), graph showing correlation between uncorrected pairwise distances of 16S rRNA and *COII* mitochondrial genes (Fig. S2), and Bayesian phylogenetic tree inferred from DNA sequences from five genes (Fig. S3).

Appendix S3 List of primers used in PCR and sequencing reactions.

BIOSKETCHES

Gerardo Hernández-Vera recently obtained his PhD at the University of East Anglia, UK, and is interested in molecular phylogenetic methods applied to studies of evolutionary ecology, biogeography, and speciation and diversification processes. Roberto Caldara, Ivo Toševski and Brent Emerson have been collaborating for several years, focusing on the taxonomy and evolutionary history of weevils within the tribe Mecinini (Curculionidae: Curculioninae).

Author contributions: B.E. conceived and led the project; R.C. and I.T. provided material and assisted with the manuscript and data collection; G.H.-V. analysed the data and wrote the manuscript; B.E. assisted with the manuscript and contributed as senior author.

Editor: Mark Carine

Table 1 Summary statistics for the five sequenced genes and the *16S* short phylogenetically informative amplicons (SPIAs) of southern African and Palaearctic lineages of endophagous weevils within the genera *Rhinusa* and *Gymnetron*. Total aligned base pairs includes outgroup taxa, the number of variable sites relates to ingroup taxa only.

	<i>COII</i>	<i>16S</i>	<i>16S</i> SPIAs	<i>AK</i>	<i>EF-1α</i>	<i>18S</i>
Aligned base pairs	697 ^a	307	150	715 ^b	647 ^c	1577
Variable sites	397	130	78	178	143	37
Parsimony informative	372 (53.4%)	104 (33.9%)	67 (44.6%)	132 (18.5%)	118 (18.2%)	30 (2%)

^a 682 bp for Gy1018

^b 423 bp for Gy976; 382 bp for Gy977

^c 418 bp for Gy975 and Gy976; 628 bp for Gy967

Table 2 Estimated times to the most recent common ancestor (TMRCA) for nodes indicating divergence of southern African and Palaearctic lineages of endophagous weevils within the genera *Rhinusa* and *Gymnetron*.

Node number	Mean (Ma)	95% HPD intervals (Ma)
I	7.4	1.1–15.9
II	11.6	4.2–20.1
III	11.0	4.1–19.1
IV	8.5	2.7–16.0

HPD, highest posterior density

FIGURE LEGENDS

Figure 1 Bayesian phylogenetic tree of southern African and Palaearctic lineages of endophagous weevils within the genera *Rhinusa* and *Gymnetron* inferred from DNA sequences from five genes (*COII*, *16S*, *EF-1 α* , *AK* and *18S*) comprising 3943 bp. Bayesian posterior probabilities and maximum likelihood bootstrap values are shown above and below branches, respectively. Seven higher order lineages and well-supported clades are labelled A–G. Species of *Gymnetron* are highlighted in bold.

Figure 2 Bayesian phylogenetic tree of southern African and Palaearctic lineages of endophagous weevils within the genera *Rhinusa* and *Gymnetron* inferred from (1) DNA sequences from five genes (*COII*, *16S*, *EF-1 α* , *AK* and *18S*) comprising 3943 bp for 54 ingroup samples and three outgroup taxa, and (2) 150 bp of nucleotide data corresponding to the *16S* short phylogenetically informative amplicons (SPIAs) sampled from 37 archival specimens and 11 degraded specimens (highlighted in grey). Species of *Gymnetron* are highlighted in bold. Green branches indicate species from southern Africa. Bayesian posterior probabilities ≥ 0.8 and maximum likelihood bootstrap values $\geq 70\%$ are shown above and below branches, respectively. Major groups are labelled A–L. Roman numerals indicate nodes for which divergence times are estimated between southern African and Palaearctic lineages (see Table 2). Pie charts indicate relative probabilities of alternative ancestral ranges mapped onto nodes of interest, (A) southern Africa, (B) Palaearctic. Branches for archival samples associated with no mutational events have been assigned zero length.

SUPPORTING INFORMATION

Molecular phylogenetic analysis of archival tissue reveals the origin of a disjunct southern African–Palearctic weevil radiation

Gerardo Hernández-Vera, Roberto Caldara, Ivo Toševski and Brent C. Emerson

Journal of Biogeography

Appendix S1 Field-sampled (a) and archival specimens (b) used in this study with GenBank accession numbers except for samples whose DNA was amplified for the short *16S* amplicons only. The full DNA sequence data matrix is available in TreeBase (<http://treebase.org/>, accession number S13611).

(a) Field-sampled specimens used in this study.

Accession	Species‡	Country of collection	GenBank accession numbers (<i>COII</i> , <i>16S</i> , <i>AK</i> , <i>18S</i> and <i>EF-1α</i> , respectively)
Gy085	<i>Rhinusa tetra</i>	Spain	JX455791, JX455766, JX091173, JX091117, JX091252
Gy091	<i>R. antirrhini</i>	Germany	JX455792, JX455767, JX091174, JX091118, HM007271
Gy135	<i>R. vestita</i>	Portugal	JX455793, JX455768, JX091175, JX091119, JX091253
Gy495	<i>R. vestita</i>	France	JX455794, JX091086, JX091176, JX091120, JX091254
Gy638	<i>R. linariae</i>	Switzerland	JX455795, JX455769, JX091177, JX091121, JX091255
Gy640	<i>R. neta</i>	Switzerland	JX455796, JX455770, JX091178, JX091122, JX091256
Gy649	<i>R. asellus</i>	Spain	JX455797, JX455771, JX091179, JX091123, JX091257
Gy658	<i>R. asellus</i>	Germany	JX455798, JX455772, JX091180, JX091124, JX091258
Gy831	<i>R. griseohirta</i>	Spain	JX455799, JX455773, JX091181, JX091125, HM007274
Gy832	<i>R. griseohirta</i>	Spain	JX455800, JX091087, JX091182, JX091126, JX091259
Gy849	<i>R. vestita</i>	Spain	EU073386, JX091088, JX091183, JX091127, JX091260
Gy868	<i>R. brondelii</i>	Serbia	JX455801, JX091089, JX091184, JX091128, JX091261
Gy871	<i>R. pilosa</i>	Romania	JX455802, JX091090, JX091185, JX091129, JX091262

Accession	Species‡	Country of collection	GenBank accession numbers (<i>COII</i> , <i>16S</i> , <i>AK</i> , <i>18S</i> and <i>EF-1α</i> , respectively)
Gy872	<i>R. collina</i>	Serbia	JX455803, JX455774, JX091186, JX091130, JX091263
Gy881	<i>R. cf. antirrhini</i> sp. 2	Serbia	HM007235, JX455775, JX091187, JX091131, JX091264
Gy886	<i>R. antirrhini</i>	UK	HM007229, JX455776, JX091188, JX091132, HM007264
Gy894	<i>R. linariae</i>	Serbia	JX455804, JX455777, JX091189, JX091133, JX091265
Gy896	<i>R. brondelii</i>	Serbia	JX455805, JX091091, JX091190, JX091134, JX091266
Gy897	<i>R. pilosa</i>	Serbia	JX455806, JX091092, JX091191, JX091135, JX091267
Gy909	<i>R. cf. antirrhini</i> sp. 2	Macedonia	HM007237, JX455778, JX091192, JX091136, HM007268
Gy912	<i>R. neta</i>	France	JX455807, JX455779, JX091193, JX091137, JX091268
Gy915	<i>R. antirrhini</i>	Spain	HM007231, JX455780, JX091194, JX091138, JX091269
Gy916	<i>R. cf. antirrhini</i> sp. 1	Serbia	HM007232, JX091093, JX091195, JX091139, HM007272
Gy917	<i>R. cf. antirrhini</i> sp. 1	Serbia	JX455808, JX455781, JX091196, JX091140, JX091270
Gy924	<i>R. bipustulata</i>	Italy	JX455809, JX455782, JX091197, JX091141, JX091271
Gy935	<i>R. neta</i>	Macedonia	JX455810, JX455783, JX091198, JX091142, JX091272
Gy938	<i>R. neta</i>	Serbia	JX455811, JX455784, JX091199, JX091143, JX091273
Gy944	<i>R. florum</i>	Serbia	HM007233, JX455785, JX091200, JX091144, HM007275
Gy949	<i>R. eversmanni</i>	Romania	JX091229, JX455786, JX091201, JX091145, JX091274
Gy959	<i>R. bipustulata</i>	Italy	JX455812, JX455787, JX091202, JX091146, JX091275
Gy984	<i>R. tetra</i>	Serbia	JX455813, JX091094, JX091203, JX091147, JX091276
Gy986	<i>R. antirrhini</i>	Serbia	HM007230, JX455788, JX091204, JX091148, JX091277
Gy996	<i>R. tetra</i>	Serbia	JX455814, JX091095, JX091205, JX091149, JX091278
Gy1024	<i>R. cf. antirrhini</i> sp. 2	Bulgaria	JX455815, JX455789, JX091206, JX091150, HM007277
283t1	<i>R. canescens</i>	Italy	JX091230, JX091096, JX091207, JX091151, JX091279

Accession	Species†	Country of collection	GenBank accession numbers (<i>COII</i> , <i>16S</i> , <i>AK</i> , <i>18S</i> and <i>EF-1α</i> , respectively)
229t1	<i>Gymnetron piceum</i>	South Africa	JX091231, JX091097, JX091208, JX091152, JX091280
229t2	<i>G. piceum</i>	South Africa	JX091232, JX091098, JX091209, JX091153, JX091281
350t1	<i>R. cf. neta</i>	Turkey	JX091233, JX091099, JX091210, JX091154, JX091282
387t1	<i>R. florum</i>	Macedonia	JX091234, JX091100, JX091211, JX091155, JX091283
413t1	<i>R. verbasci</i>	Macedonia	JX091235, JX091101, JX091212, JX091156, JX091284
430t1	<i>R. linariae</i>	Turkey	JX091236, JX091102, JX091213, JX091157, JX091285
484t1	<i>R. verbasci</i>	Macedonia	JX091237, JX091103, JX091214, JX091158, JX091286
442t2	<i>R. cf. neta</i>	Turkey	JX091238, JX091104, JX091215, JX091159, JX091287
303i	<i>R. collina</i>	Turkey	JX091239, JX091105, JX091216, JX091160, JX091288
401i	<i>R. eversmanni</i>	Serbia	JX091240, JX091106, JX091217, JX091161, JX091289
423i	<i>R. canescens</i>	Italy	JX091241, JX091107, JX091218, JX091162, JX091290
596i	<i>R. linariae</i>	Turkey	JX091242, JX091108, JX091219, JX091163, JX091291
631i	<i>R. melas</i>	Serbia	JX091243, JX091109, JX091220, JX091164, JX091292
632i	<i>R. melas</i>	Serbia	JX091244, JX091110, JX091221, JX091165, JX091293
644i	<i>R. florum</i>	Turkey	JX091245, JX091111, JX091222, JX091166, JX091294
Gy975	<i>G. melanarium</i>	UK	JX091246, JX091112, JX091223, JX091167, JX091295
Gy976	<i>G. veronicae</i>	Italy	JX091247, JX091113, JX091224, JX091168, JX091296
Gy977	<i>G. villosulum</i>	Italy	JX091248, JX091114, JX091225, JX091169, JX091297
Gy1138	<i>G. rotundicolle</i>	Italy	
Gy1139	<i>G. rotundicolle</i>	Italy	
Gy1140	<i>G. rotundicolle</i>	Italy	
Gy1146	<i>G. rotundicolle</i>	Italy	
Gy1145	<i>G. rotundicolle</i>	Italy	
Gy1144	<i>G. rotundicolle</i>	Italy	
Gy1143	<i>G. rotundicolle</i>	Italy	
Gy1142	<i>G. rotundicolle</i>	Italy	
Gy1141	<i>G. rotundicolle</i>	Italy	

Accession	Species‡	Country of collection	GenBank accession numbers (<i>COII</i> , <i>16S</i> , <i>AK</i> , <i>18S</i> and <i>EF-1α</i> , respectively)
Gy1137	<i>G. rotundicolle</i>	Italy	
Gy1147	<i>G. rostellum</i>	Italy	
Gy967	<i>Cleopomiarus meridionalis</i>	Italy	JX091249, JX091115, JX091226, JX091170, JX091298
§ Gy1008	<i>Ceutorhynchus scrobicollis</i>	Germany	JX091250, JX091116, JX091227, JX091171, JX091299
§ Gy1018	<i>Tychius junceus</i>	Spain	JX091251, JX455790, JX091228, JX091172, JX091300

§ Outgroup taxa.

*Samples with comparatively weak PCR products that did not generate readable sequence chromatograms.

‡ Specimens are deposited within the collection of Roberto Caldara.

(b) Archival specimens used in this study.

Accession	Species	Country	Year of collection
Gy1036	<i>G. gossypinus</i>	South Africa	N.A.
Gy603	<i>R. herbarum</i>	N.A.	N.A.
Gy604	<i>R. moroderi</i>	N.A.	N.A.
Gy605	<i>G. villosulum</i>	N.A.	N.A.
Gy1032	<i>R. comosa</i>	N.A.	N.A.
Gy1034	<i>R. herbarum</i>	France	N.A.
Gy1037	<i>G. perrinae</i>	South Africa	N.A.
Gy1149	<i>R. scrophulariae</i>	United Arab Emirates	N.A.
Gy1148	<i>R. scrophulariae</i>	United Arab Emirates	N.A.
T1	<i>G. bipartitum</i>	South Africa	1985
T3	<i>R. mauritii</i>	Morocco	2008
T4	<i>R. depressa</i>	Morocco	N.A.
T5	<i>G. vittipenne</i>	Armenia	2001
T6	<i>R. comosa</i>	Turkey	1986
T2	<i>G. bisignatum</i>	South Africa	1987
6D	<i>G. bisignatum</i>	South Africa	1990
2D	<i>R. exigua</i>	Turkey	2003
3D	<i>G. hystrix</i>	South Africa	1991
4D	<i>G. hystrix</i>	South Africa	1991
5D	<i>G. agile</i>	South Africa	2002
7D	<i>G. buddleiae</i>	South Africa	1993
9D	<i>G. minimum</i>	South Africa	2006
10D	<i>G. pauxillum</i>	South Africa	2005
11D	<i>G. quadratum</i>	South Africa	2009
13D	<i>G. aequale</i>	Turkey	1999
14D	<i>G. desbrochersi</i>	Russia	1990
15D	<i>G. linkei</i>	Turkey	2002
16D	<i>G. stimulosum</i>	Czech Republic	1954
17D	<i>G. tibiellum</i>	Italy	1997
18D	<i>G. vittipenne</i>	Armenia	1987
19D	<i>R. algerica</i>	Tunisia	2006
20D	<i>R. brisouti</i>	Kazakhstan	2000
21D	<i>R. emmrichi</i>	Turkmenistan	1991
23D	<i>R. mateui</i>	Tunisia	1998
T14	<i>R. exigua</i>	Turkey	1996
Gy1033	<i>R. moroderi</i>	N.A.	N.A.
Gy974	<i>G. clepsydra</i>	South Africa	N.A.
*Gy1035	<i>G. oxystomoides</i>	South Africa	N.A.
*1D	<i>R. dieckmanni</i>	Bulgaria	1958
*8D	<i>G. bipartitum</i>	South Africa	1986
*12D	<i>G. simulator</i>	South Africa	1983
*22D	<i>R. littorea</i>	France	N.A.

N.A. Not available.

Appendix S2 Supplementary figures.

Figure S1 Leg of *Gymnetron villosulum* remounted on an entomological card after DNA extraction.

Figure S2 Graph of the correlation between uncorrected pairwise distances of the mitochondrial genes 16S ribosomal RNA (*16S*) and cytochrome *c* oxidase subunit II (*COII*).

Figure S3 Bayesian phylogenetic tree of southern African and Palaearctic lineages of endophagous weevils within the genera *Rhinusa* and *Gymnetron* inferred from a data set of five gene DNA sequences (*COII*, *16S*, *EF-1 α* , *AK* and *18S*) comprising 3943 bp. Nucleotide data for highlighted taxa were reduced to 150 bp corresponding to the *16S* short phylogenetically informative amplicons (SPIAs). Posterior probabilities are shown above branches and major groups are indicated (A–G).

Appendix S3 Primers used in the amplification of nuclear and mitochondrial gene fragments: arginine kinase (*AK*), 18S ribosomal RNA (*18S*), elongation factor-1 α (*EF1- α*), cytochrome *c* oxidase subunit II (*COII*) and 16S ribosomal RNA (*16S*).

Gene	Name of primer	Sequence	Amplicon length (bp)
<i>AK</i>	ArgKforB2	5'-GAYTCCGGWATYGGWATCTAYGCTCC (f)	774
	ArgKrevB1	5'-TCNGTRAGRCCCATWCGTCTC (r)	
	ArgKG1f	5'-ATYGGWATCTAYGCTCCYGAYGC (f)	757
	ArgKG1r	5'-GCCCATWCGTCTCTTRTTRGAAAT (r)	
<i>18S</i>	18S5'	5'-GACAACCTGGTTGATCCTGCCAGT (f)	1146
	18Sb0.5	5' –GTTTCAGCTTTGCAACCAT (r)	
	18Sa1.0	5' –GGTGAAATTCTTGACCGTC (f)	892
	18S3'I	5' –CACCTACGGAAACCTTGTTACGAC (r)	
<i>EF-1 α</i>	EF1-Bf	5'-AGAACGTGAACGTGGTATCA (f)	877–1112
	EF-Br	5'-CTTGGAGTCACCAGCTACATAACC (r)	
<i>COII</i>	TL2-J-3038	5'-TAATATGGCAGATTAGTGCATTGGA (f)	739
	TK-N 3782	5'-GAGACCATTACTTGCTTTCAGTCATCT (r)	
<i>16S</i>	16Sar	5'-CGCCTGTTTATCAAAAACAT (f)	518
	16Sbr	5'-CCGGTCTGAACTCAGATCACGT (r)	
	16S_7bp_FGer	5'-[GTAAAACGACGGCCAGT]AATMATTAGTTTYTTAATT (f)	95
	16S_7bp_RGer	5'-TAYAGGGTCTTCTCGTCTT (r)	
	16S_48bpF1	5'-CGAGAAGACCCTATAGAGTTT (f)	55
	16S_48bpR1	5'-TCAATCACCCCAAYYAAAT (r)	

(f) = forward reading, (r) = reverse reading. [] = M13 adaptor sequence.