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Phylogenetic analyses of band-winged grasshoppers (Orthoptera, Acrididae, Oedipodinae) reveal convergence of wing morphology

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Historically, morphological traits have been used to examine the relationships of distantly related taxa with global distributions. The use of such traits, however, may be misleading and may not provide the resolution needed to distinguish various hypotheses that may explain the distribution patterns of widely distributed taxa. One such taxon, the Oedipodine grasshoppers, contains two tribes principally defined by wing morphologies: the Bryodemini have broad wings whereas Sphingonotini are narrow-winged. Through the use of morphological features alone, hypotheses concerning the evolution of these tribes cannot be distinguished. To differentiate hypotheses that may explain the distribution of Oedipodines, such as vicariance, natural dispersal and anthropogenic translocation, we used two mitochondrial and three nuclear gene fragments to reconstruct the phylogenetic relationships within and between the two tribes, and employed a molecular clock to evaluate the hypotheses of vicariance and dispersal. Our results clearly reject monophyly of the tribes and revealed monophyletic Old and New World clades, which is in agreement with previous molecular studies. The split between both clades was dated at 35 Ma (±12 Ma). This clearly rejects the vicariance hypothesis and supports a single invasion via the Beringian land bridge. In addition, our data clearly show that the similarities in wing morphology used for distinguishing both tribes are the result of at least one convergent event. Our study shows that interpretations of relationships based on the currently accepted taxonomy in the study groups will be error prone. We suggest that future revisions that consider our findings are required.

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Introduction

A central aim of phylogeography is to understand how geographic events affected the evolution of current biodiversity and distribution of biota (Avise 2004). Many animal and plant taxa have Holarctic distributions and occupy similar habitats in the Old and the New World. These patterns can be explained in a variety of ways including vicariance, natural dispersal and anthropogenic translocation (Carranza & Arnold 2003; Zhou *et al.* 2006). The plausibility of these different hypotheses depends on the time scale when phylogenetic splits between Palaeartic and Nearctic taxa occurred: If the timing of the phylogenetic split correlates with the separation of Laurentia and Eurasia (90–65 Ma; Sanmartín *et al.* 2001), one can infer that the taxon was originally widespread in Pangaea and gene flow was disrupted after the continental plates shifted (Archibald 2003; Diogo 2004; Wolfe *et al.* 2005). If the phylogenetic split occurred considerably later, dispersal is a more likely cause of the current distribution. Long-distance dispersal between Eurasia and North America could have occurred via rafting (Houle 1998), flight (Lovejoy *et al.* 2006), or across land bridges that have connected

these two land masses many times since the break-up of Pangea (Sanmartín *et al.* 2001). Between 55–50 Ma the Thulean Land Bridge represented a viable migration route across the North Atlantic Ocean (Sanmartín *et al.* 2001). Approximately 100 Ma, the Beringian Land Bridge formed the first time and supported migration across the Northern Pacific Ocean. This connection finally broke apart about 5.5–5.4 Ma; however, it appears to have reformed several times during the Pleistocene (Sanmartín *et al.* 2001). The Beringian Land Bridge has been suggested to be the colonization route for a variety of taxa (Townsend *et al.* 2011).

Due to these manifold possibilities of biotic exchange between North America and Eurasia, Holarctic distribution patterns are rather common. It is thus not surprising that many higher taxa share representatives on both continents. However, the phylogenetic relationships among such taxa have not been tested with modern molecular tools and their systematic relationships might additionally be blurred by convergence. This might be particularly true for higher taxa erected by early taxonomists, who used morphological characters for identifying taxonomic units. As morphological traits are often strongly affected by natural selection, one might also expect to find similar morphologies in related habitats at distant locations as result of convergent evolution (Moore & Willmer 1997). While phylogenetic relationships derived from morphological data often proved to be correct (e.g. Whalen & Caruso 1983; Brown *et al.* 1994; Cameron & Williams 2003), to ultimately distinguish common ancestry from convergence, an analysis of selectively neutral characters is needed. Molecular markers provide selectively neutral characters that can be used to understand the phylogenetic relationships of taxa and to evaluate different biogeographic hypotheses. Due to its fast evolutionary rates, mitochondrial DNA was long the single preferred genetic marker system in phylogeography (Avise 2004). However, recent studies have shown that the maternal heredity of mtDNA can also lead to misinterpretation of phylogenetic relationships caused by introgression events (Shaw 2002; Funk & Omland 2003). Mitochondrial DNA also saturates more quickly making it difficult to study deep phylogenetic relationships (Brandley *et al.* 2011). Combined analyses of mitochondrial and nuclear DNA can overcome these limitations and often reveal more complete pictures of phylogenies.

In addition, the analysis of DNA sequences can be used to generate rough estimates of time since divergence of lineages by utilizing a molecular clock approach. The use of molecular clocks aids discriminating different phylogeographic hypotheses by comparing the timing of phylogenetic and geographic events, such as the availability of migration routes. Due to the long periods between the availability of different migration routes for Holarctic taxa, even time since divergence estimates that have large variances can be useful in discriminating phylogeographic hypotheses (Carranza & Arnold 2003).

Band-winged grasshoppers (Oedipodinae) are one of the few nearly globally distributed subfamilies within the short-horned grasshoppers (Caelifera), with major radiations in the Palaearctic as well as the Nearctic (Eades *et al.* 2011). Within the Oedipodinae, the tribes Sphingonotini and Bryodemini are closely related and a recent phylogenetic study suggests that they may be sister groups (Fries *et al.* 2007). Both tribes occur throughout the Holarctic, with a considerable number of species in both hemispheres. Genera have been assigned to these tribes mainly according to similarities in their wing morphology (Otte 1984; Fries *et al.* 2007). Bryodemini are characterized by broad fore wings and triangular hind wings often with thickened veins, while Sphingonotini have much more slender fore and hind wings. However, a recent phylogenetic study rejected the monophyly of both tribes and found Nearctic genera of both groups clustering together (Fries *et al.* 2007), which supports results from older cytological studies (White 1948, 1950, 1973).

The aim or our study was to evaluate the intercontinental relationships within Bryodemini and Sphingonotini from the Palaearctic and the Nearctic and test the alternative hypotheses of vicariance vs. dispersal (Fig. 1). We sampled and analysed sequences of two mitochondrial and three nuclear genes of both tribes over major parts of the Holarctic. Furthermore, we used phylogenetic and evolutionary analyses to determine whether morphological similarities of Palaearctic and Nearctic taxa are due to

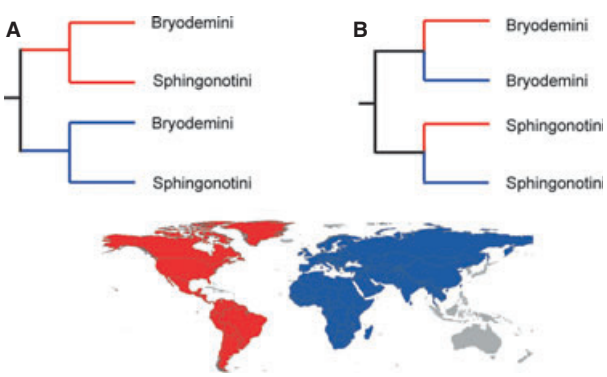

Fig. 1 Two main hypotheses for the relationships among bandwind grasshoppers: (a) single vicariance or colonization event followed by convergent evolution of wing morphology or, (b) multiple colonization dispersal or vicariance in each tribe and common ancestry of wing morphology.

common ancestry or convergent evolution. In detail we want to test the following hypotheses:

- The morphological similarities within the tribes Bryodemini and Sphingonotini are caused by convergent evolution rather than by common ancestry. Thus, the current systematic status of these taxa does not reflect their phylogenetic relationships.
- The current distribution of Bryodemini and Sphingonotini has been caused by natural dispersal rather than by vicariance.

Material and methods

Study objects

Both tribes, Sphingonotini and Bryodemini, belong to the subfamily Oedipodinae. The Sphingonotini Johnston, 1956 are the most species-rich tribe within the Oedipodinae. Currently, the Sphingonotini contain 279 species in 20 genera which are distributed over all major continents (except for Antarctica). In contrast, the tribe Bryodemini Bei-Bienko, 1930 is less diverse with 57 species in eight genera. Most Bryodemini occur in the eastern Palaearctic. However, a limited number of species can be found in the western Palaearctic and the Orientalis (Eades *et al.* 2011). Only the genus *Circotettix* occurs in the Nearctic. Representatives of Sphingonotini are mostly found in deserts, semi-deserts and other arid habitats, while Bryodemini mainly occur in mountainous habitats.

Species within the tribes share similar morphological features. Most Sphingonotini are smaller in overall body size and have narrow fore and hind wings. Bryodemini are generally larger, have a stouter body and share a distinct wing morphology. Their forewings are leathery and relatively wide, while the hind wings are triangular and have thickened vannal veins (Benediktov 1998).

Despite their similarity in wing morphology, previous cytological and genetic studies suggest that the tribes are not monophyletic. These studies suggested that some Nearctic genera, including the Sphingonotini *Trimerotropis*, *Dissosteira* and *Spharagemon* and the Bryodemini *Circotettix* form a monophyletic group (White 1948, 1950, 1973; Fries *et al.* 2007). This would mean that the morphological similarities are the result of convergent evolution. However, this finding has yet to impact the taxonomic organization of Oedipodines.

Sampling

Fifty-five individuals from the tribes Sphingonotini and Bryodemini belonging to eight genera and 38 species were collected over parts of Asia, Africa, Europe, North and South America (see Table 1 for details). One species from Argentina is currently undescribed. As outgroup we used one specimen each of *Chortophaga viridifasciata*, which belongs to a different Oedipodinae tribe (Chortophagini) and one species belonging to a separate subfamily, the Gomphocerinae *Cibolacris parviceps*. Specimens were caught by hand or with sweep nets, killed and stored either at −20 °C, in 99.8% ethanol p.a., or as dried museum material.

DNA extraction and gene sequencing

Genomic DNA was extracted from femoral muscle tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol for tissue samples. We amplified two mitochondrial and three nuclear gene fragments. Primers for the mitochondrial NADH dehydrogenase subunit 5 (ND5) were obtained from Su *et al.* (1998) and Hochkirch (2001). The nuclear Internal Transcribed Spacer 2 (ITS2) and Histone 3 (H3) were amplified using primers provided by Tautz *et al.* (1988) and Colgan *et al.* (1998). In addition to these two fragments we designed primers for the mitochondrial Cytochrome Oxidase I gene (COI), and for a fraction of the nuclear ribosomal 18s gene. These primers were designed using the online program PRIMER3 v. 0.4.0 (Rozen & Skaletsky 2000) based on several Oedipodines and other Orthoptera sequences obtained from Genbank. Primer sequences are provided in Table 2.

PCR reactions were performed using three different polymerases (DyNAzyme by Finnzymes; HotMasterMix by 5Prime; HotStarTaq Master Mix by Qiagen). The following setup was used for DyNAzyme (Finnzymes, Vantaa, Finland Integrated DNA technologies, Coralville, IA, USA): 36.6 μL of diH_2O, 6 μL of 10× PCR buffer (reaction concentration 1×), 4.8 μL of dNTP mixture (0.2 μM each), 0.6 μL of DyNAzyme™ DNA Polymerase (1.2 U), 3 μL of each primer (0.5 μM, Integrated DNA Technologies, USA) and 6 μL of DNA template adding up to a total volume of 60 μL. Amplification conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min denaturation, 1 min annealing (ND5 48 °C, COI 53–43 °C, H3 57 °C, ITS2 60–55 °C, 18S 58 °C) and 72 °C for 2 min elongation, with a final elongation step at 72 °C for 10 min. For HotMasterMix (5Prime) the following PCR conditions were used: 26 μL of diH_2O, 20 μL of HotMasterMix, 1.25 μL of each primer and 1.5 μL of DNA template adding up to a total volume of 50 μL. Amplification conditions were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min denaturation, 1 min annealing (45 °C) and 65 °C for 1 min elongation, with a final elongation step at 65 °C for 10 min. For HotStarTaq Master Mix (Qiagen) we used the following PCR conditions: 26 μL of diH_2O, 20 μL of HotStarTaq Master Mix, 1.25 μL of each primer and 1.5 μL of DNA template adding up to a total volume of 50 μL.

Table 1 Overview of samples used for analyses. Given is the sample ID, taxonomic assignment, country, collectors and Genbank accession numbers which correspond with gene sequences deposited at NCBI Genbank

						Genbank accession nos.				
ID	Tribe	Genus	Species	Country/Island	Collected by	ND5	COI	H3	ITS2	18S
T15	Sphingonotini	*Conozoa*	*texana*	USA, NM	D. Ferguson	JQ286632	JQ286500	JQ286567	JQ286699	JQ512994
K379	Sphingonotini	*Leptopternis*	*maculatus*	Tunisia	A. Hochkirch	JQ513140	JQ513074	JQ513183	JQ513094	JQ512995
K405	Sphingonotini	*Sphingonotus*	*azurescens*	Spain	A. Hochkirch	JQ513141	JQ513072	JQ513184	JQ513095	JQ512996
K315	Sphingonotini	*Sphingonotus*	*caerulans*	France	A. Hochkirch	JQ513142	JQ513068	JQ513185	JQ513096	JQ512997
K608	Sphingonotini	*Sphingonotus*	*caerulans*	Finland	A. Hochkirch	JQ513143	JQ513067	JQ513186	JQ513097	JQ512998
K512	Sphingonotini	*Sphingonotus*	*canariensis*	Cape Verde	M. Lecoq	JQ513144	JQ513077	JQ513187	JQ513098	JQ512999
K403	Sphingonotini	*Sphingonotus*	*candidus*	Sardinia	Y. Görzig	JQ513145	JQ513066	JQ513188	JQ513099	JQ513000
K90	Sphingonotini	*Sphingonotus*	*femoralis*	Niger	T. McNary	JQ513146	JQ513065	JQ513189	JQ513100	JQ513001
K383	Sphingonotini	*Sphingonotus*	*finotianus*	Tunisia	A. Hochkirch	JQ513147	JQ513073	JQ513190	JQ513101	JQ513002
K456	Sphingonotini	*Sphingonotus*	*fuerteventurae*	Fuerteventura	A. Hochkirch	JQ513148	JQ513071	JQ513191	JQ513102	JQ513003
K014	Sphingonotini	*Sphingonotus*	*guanchus*	Gran Canary	A. Hochkirch	EU266743	JQ513064	JQ513192	JQ513103	JQ513004
K638	Sphingonotini	*Sphingonotus*	*guanchus*	Gran Canary	R. Bland	JQ513149	JQ513063	JQ513193	JQ513104	JQ513005
K651	Sphingonotini	*Sphingonotus*	*maroccanus*	Morocco	M. Husemann	JQ513150	JQ513075	JQ513194	JQ513105	JQ513006
K616	Sphingonotini	*Sphingonotus*	*ningsianus*	China	Li Xinjiang	JQ513151	JQ513060	JQ513195	JQ513106	JQ513007
K470	Sphingonotini	*Sphingonotus*	*octofasciatus*	Tunisia	A. Hochkirch	JQ513152	JQ513058	JQ513196	JQ513107	JQ513008
K351	Sphingonotini	*Sphingonotus*	*rubescens*	Fuerteventura	A. Hochkirch	JQ513153	JQ513069	JQ513197	JQ513108	JQ513009
K510	Sphingonotini	*Sphingonotus*	*rubescens*	Cape Verde	M. Lecoq	JQ513154	JQ513070	JQ513198	JQ513109	JQ513010
K150	Sphingonotini	*Sphingonotus*	*savignyi*	Tenerife	A. Hochkirch	JQ513155	JQ513076	JQ513199	JQ513110	JQ513011
K214	Sphingonotini	*Sphingonotus*	*scabriculus*	Namibia	W. Schuett	JQ513156	JQ513061	JQ513200	JQ513111	JQ513012
K615	Sphingonotini	*Sphingonotus*	*tsilingensis*	China	Li Xinjiang	JQ513157	JQ513059	JQ513201	JQ513112	JQ513013
T80	Sphingonotini	*Sphingonotus*	*turkanae*	Kenya	C. Hemp	–	JQ513062	JQ513202	JQ513113	JQ513014
K227	Sphingonotini	*Thalpomena*	*coerulescens*	Morocco	A. Hochkirch	JQ513158	JQ513057	JQ513203	JQ513114	JQ513017
K641	Sphingonotini	*Thalpomena*	*viridipennis*	Morocco	A. Hochkirch	JQ513159	JQ513056	JQ513204	JQ513115	JQ513015
T28	Sphingonotini	*Trimerotropis*	*californicus*	USA, NM	D. Ferguson	JQ513160	JQ513048	JQ513205	JQ513116	JQ513016
T17	Sphingonotini	*Trimerotropis*	*cyaneipennis*	USA, NM	D. Ferguson	JQ513161	JQ513040	JQ513206	JQ513117	JQ513018
T105	Sphingonotini	*Trimerotropis*	*pallidipennis*	USA, MT	R. D. Scott	JQ513162	JQ513036	JQ513207	JQ513118	JQ513019
T110	Sphingonotini	*Trimerotropis*	*latifasciata*	USA, MT	R. D. Scott	JQ513163	JQ513047	JQ513208	JQ513119	JQ513020
T1	Sphingonotini	*Trimerotropis*	*maritima*	USA, TX	M. Husemann	JQ286630	JQ286498	JQ286565	JQ286698	JQ513032
T52	Sphingonotini	*Trimerotropis*	*maritima*	USA, TX	M. Husemann	JQ286629	JQ286497	JQ286564	JQ286697	JQ513021
T116	Sphingonotini	*Trimerotropis*	*ochraceipennis*	Chile	J. Pizarro	JQ286681	JQ286549	JQ286607	JQ286725	JQ513022
T119	Sphingonotini	*Trimerotropis*	*ochraceipennis*	Chile	J. Pizarro	JQ286680	JQ286548	JQ286606	JQ286724	JQ513023
T128	Sphingonotini	*Trimerotropis*	*ochraceipennis*	Chile	J. Pizarro	JQ286688	JQ513038	JQ286622	JQ286736	JQ513024
T156	Sphingonotini	*Trimerotropis*	*pallidipennis*	Mexico	D. Salas Araiza	JQ286654	JQ286522	JQ286581	–	JQ513025
T162	Sphingonotini	*Trimerotropis*	*pallidipennis*	Mexico	D. Salas Araiza	JQ286669	JQ286537	JQ286596	–	JQ513026
T163	Sphingonotini	*Trimerotropis*	*pallidipennis*	Mexico	D. Salas Araiza	JQ286667	JQ286535	JQ286594	–	JQ513027
T31	Sphingonotini	*Trimerotropis*	*pistrinaria*	USA, NM	D. Ferguson	JQ513165	JQ513046	JQ513210	JQ513121	JQ513029
T124	Sphingonotini	*Trimerotropis*	*cf pistrinaria*	USA, TX	M. Husemann	JQ513164	JQ513035	JQ513209	JQ513120	JQ513028
T132	Sphingonotini	*Trimerotropis*	*saxatilis*	USA, TX	M.Hanitzsch	JQ286635	JQ286503	JQ286570	JQ286700	JQ513030
T71	Sphingonotini	*Trimerotropis*	*sp*	Argentina	V. Confalonieri	JQ286685	JQ513037	JQ286611	JQ286728	JQ513031
Li1	Bryodemini	*Bryodema*	*diamesum*	China	Li Xinjiang	JQ513122	JQ513049	JQ513166	JQ513079	JQ512974
Li2	Bryodemini	*Bryodema*	*diamesum*	China	Li Xinjiang	JQ513123	JQ513050	JQ513167	JQ513080	JQ512975
S10-3	Bryodemini	*Bryodema*	*gebleri*	Russia	M. Husemann	JQ513124	–	JQ513168	JQ513081	JQ512976
S3-3	Bryodemini	*Bryodema*	*gebleri*	Russia	M. Husemann	JQ513125	JQ513051	JQ513169	JQ513082	JQ512977
S8-1	Bryodemini	*Bryodema*	*gebleri*	Russia	M. Husemann	JQ513126	JQ513052	–	JQ513083	JQ512978
MS1	Bryodemini	*Bryodemella*	*holdereri*	Russia	M. G. Sergeev	JQ513127	JQ513053	JQ513170	JQ513078	JQ512979
S15-5	Bryodemini	*Bryodemella*	*holdereri*	Russia	M. Husemann	JQ513128	–	JQ513171	JQ513084	JQ512980
S3-1	Bryodemini	*Bryodemella*	*tuberculata*	Russia	M. Husemann	JQ513129	JQ513055	JQ513172	JQ513085	JQ512981
S4-4	Bryodemini	*Bryodemella*	*tuberculata*	Russia	M. Husemann	JQ513130	–	JQ513173	JQ513086	JQ512982
Sw1	Bryodemini	*Bryodemella*	*tuberculata*	Sweden	M. Husemann	JQ513131	JQ513054	JQ513174	JQ513087	JQ512983
T25	Bryodemini	*Circotettix*	*cf. maculatus*	USA, CA	D. Ferguson	JQ513134	JQ513041	JQ513177	–	JQ512987
T26	Bryodemini	*Circotettix*	*cf. maculatus*	USA, CA	D. Ferguson	JQ513135	JQ513045	JQ513178	JQ513089	JQ512988
T9	Bryodemini	*Circotettix*	*rabula*	USA, NM	D. Ferguson	JQ286650	JQ286518	JQ286577	JQ286709	JQ512990
T10	Bryodemini	*Circotettix*	*rabula*	USA, NM	D. Ferguson	JQ286651	JQ286519	JQ286578	JQ286710	JQ512989
T108	Bryodemini	*Circotettix*	*cf. rabula*	USA, MT	R. D. Scott	JQ513136	JQ513044	JQ513179	JQ513090	JQ512986

Table 1 Continued

ID	Tribe	Genus	Species	Country/Island	Collected by	Genbank accession nos.				
						ND5	COI	H3	ITS2	18S
T150	Bryodemini	*Circotettix*	*stenometopus*	USA, CA	D. Ferguson	JQ513137	JQ513039	JQ513180	JQ513091	JQ512991
T23	Bryodemini	*Circotettix*	*undulatus*	USA, CA	D. Ferguson	JQ513138	JQ513043	JQ513181	JQ513092	JQ512992
T24	Bryodemini	*Circotettix*	*undulatus*	USA, CA	D. Ferguson	JQ513139	JQ513042	JQ513182	JQ513093	JQ512993
T76	Chortophagini	*Chortophaga*	*viridifasciata*	USA, TX	M. Husemann	JQ513132	JQ513034	JQ513175	JQ513088	JQ512984
T112	Cibolacrini (Gomphocerinae)	*Cibolacris*	*parviceps*	USA, TX	M. Husemann	JQ513133	JQ513033	JQ513176	–	JQ512985

Table 2 Sequences of primers used

Name	Gene	Sequence	References
ND5-His	ND5	CCT GTT TCT GCT TTA GTT CA	Su *et al.* (1998)
Nd5-Phe	Nd5	GTC ATA CTC TAA ATA TAA GCT A	Su *et al.* (1998)
ITS3	ITS2	GCA TCG ATG AAG AAC GCA GC	Tautz *et al.* (1988)
ITS4	ITS2	TCC TCC GCT TAT TGA TAT GC	Tautz *et al.* (1988)
H3AF	H3	ATG GCT CGT ACC AAG CAG ACV GC	Colgan *et al.* (1998)
H3AR	H3	ATA TCC TTR GGC ATR ATR GTG AC	Colgan *et al.* (1998)
COI-F	COI	CCA TCT TAC CGC AAA AAT GAT	This study
COI-R	COI	CTG GGT GWC CAA AGA ATC AAA	This study
18S-F1	18S	ACG AAC AGA AGC GAA AGC AT	This study
18S-R1	18S	ATC CCT AGC ACG AAG GAG GT	This study

Amplification conditions were: 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min denaturation, 1 min annealing (45 °C) and 72 °C for 1 min elongation, with a final elongation step at 72 °C for 10 min.

PCR products were visualized on a 1% agarose gel stained with Gel Red (0.1×, Biotium, Hayward, CA, USA) or with SYBR Green I (Biozym, Hessisch, Oldendorf, Germany). The product was purified using either Solid-phase Reversible Immobilization (SPRI, DeAngelis *et al.* 1995) with carboxylated magnetic beads (Bangs Laboratories, Fishers, IN, USA) and a 96-Ring SPRIplate (Agencourt, Beverly, MA, USA) or Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were either sequenced at the Yale Sequencing Facility (New Haven, CT, USA) using the BIG DYE TERMINATOR 3.1 cycle sequencing kit on a 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or on a MEGABACE 1000 automated sequencer at the University of Trier with the DYEnamic ET Terminator Cycle Sequencing Premixkit (GE Healthcare, Munich, Germany). Sequences were deposited in Genbank (see Table 1).

Sequence analysis

Sequences were inspected, trimmed and aligned using GENEIOUS 5.0.3 (Drummond *et al.* 2011). General statistics were calculated with DNASP v. 5.10 (Librado & Rozas 2009). A species tree incorporating all gene fragments was generated using *BEAST (Heled & Drummond 2010) as implemented in the BEAST package v.1.6.1 (Drummond & Rambaut 2007). The input file for BEAST was created with BEAUti v.1.6.1. Species were defined according to current taxonomy unless the sample is currently undescribed (one *Trimerotropis* species from Argentina). We determined the best-fitting substitution models for each gene fragment using MRMODELTEST v. 2.3 (Nylander 2004). The GTR+I+G model was chosen for the mitochondrial gene fragments (COI and ND5), which were treated as linked partitions. The GTR+I+G model was used in the analysis of H3. JC was chosen for the 18S fragment, but HKY was used in *BEAST as it represents the simplest model implemented in the program. HKY+I+G was used for ITS2. A speciation Yule process was used as tree prior. We ran the analysis for 50 million generations sampling trees every 1000 generations. The resulting log-file was imported into TRACER v1.5 (Rambaut & Drummond 2009) and used to estimate the appropriate burn-in phase and test for convergence of the chains. A consensus tree was generated using TREEANNOTATOR v.1.6.1 discarding a burn-in of 10% (implemented in the BEAST package v.1.6.1). The tree was visualized using FIGTREE v. 1.3.1 (Rambaut 2011).

In addition we performed a Bayesian analyses on the concatenated dataset. The dataset was partitioned and the same substitution models as for *BEAST analysis were used for the partitions. We used MRBAYES v.3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) and ran the analysis for 50 million generations, sampling trees every 1000 generations. A burn-in of 5000 trees (10%) was discarded before calculating a consensus tree that was then visualized using FIGTREE v. 1.3.1

We tested our two major hypotheses (Fig. 1) and a third hypothesis resulting from the concatenated analysis using a Bayes factors comparison (Kass & Raftery 1995; Marek & Bond 2006). Bayes factors analyses differ from more traditional hypotheses testing methods in not offering criteria for the absolute rejection of a null hypotheses, but instead it allows evaluating alternative hypotheses (Kass & Raftery 1995). Bayes factors are twice the difference in harmonic means of log-likelihoods between constrained and unconstrained trees. We constrained our phylogenies

to the topologies representing the respective hypothesis and ran *BEAST with these constrained clades. We defined the topology generated by an unconstrained run as hypothesis, with four major groups being predefined as units (Nearctic Bryodemini including the genus *Circotettix*), Nearctic Sphingonotini (including the genera *Trimerotropis* and *Conozoa*), Palaearctic Bryodemini (including the genera *Bryodema* and *Bryodemella*), Palaearctic Sphingonotini (including the genera *Sphingonotus*, *Thalpomena*, and *Leptopternis*). For the alternative hypotheses the same units were predefined, but the analyses was further constrained to reflect the respective hypothesis tested: H_1: the four groups cluster by their geographical distribution (Fig. 1A), H_2: the groups cluster by their wing morphology and current systematic position (Fig. 1B), H_3: the tree topology was constrained to reflect the intergroup relationships recovered in the concatenated analysis (Fig. 3). The log-files from these analyses were then used as input for TRACER v.1.5.0, which compares the likelihood scores for each hypothesis with the unconstrained hypothesis, and generates Bayes factors. The hypothesis being most similar to the unconstrained topology (indicated by a low Bayes factor) is considered the best. The Bayes factor approach has been employed in a variety of phylogenetic studies and has represented a valuable tool when *a priori* hypotheses need to be compared (Suchard *et al.* 2005; Marek & Bond 2006; Genner & Turner 2012).

In order to reconstruct the evolution of the two wing phenotypes we used the Maximum Parsimony approach implemented in RASP v.1.107 (Yu *et al.* 2010). The maximum number of states was fixed to one while the extinction weight was set to 50%. Divergence times were estimated using a molecular clock approach as implemented in BEAST. We used the concatenated mtDNA dataset for dating (55 sequences, 1715 bp) and employed a 'relaxed clock: Uncorrelated log-normal' distribution with three calibration points. First, the only known fossil, *Bryodema croatica* Zeuner, 1942, for the tribes of interest was used for tree calibration. It has been dated to the lower Miocene (23.03–15.97 Ma). Hence, the node was calibrated using a normal distribution with a mean at 19.95 Ma and a standard deviation of 2. We used two well documented biogeographic events as additional calibration points: (i) the split between the South American and North American lineages of the *Trimerotropis pallidipennis* group, which is correlated with the opening of the Panama Isthmus 3.5–4 Ma (3.75 ± 0.1 SD) and (ii) the age of Gran Canaria (~15 Myr), which is used as an estimate of the maximum age of the island endemic *S. guanchus*. We used a 'Speciation: Yule process' tree prior. The analysis was run for 50 million generations logging trees every 1000 generations. Trees were summarized with TREEANNOTATOR v.1.6.1 after discarding a burn-in of 10% and the resulting consensus tree was visualized with FIGTREE v. 1.3.1. Node ages are shown together with 95% highest posterior density bars indicating a range of age estimates.

It is important to note that due to the shortage of precise calibration points from external and independent data (e.g. dated fossil records, known biogeographic events, or paleoclimatic reconstructions), or due to the heterogeneity rate of evolution between the calibrated and uncalibrated branches, temporal estimates presented here are very rough and should be treated with caution. However, on the large temporal scales the different hypotheses are based on (vicariance vs. dispersal) these estimates are expected to be robust.

Results

We analysed two mitochondrial and three nuclear gene fragments; the mitochondrial COI dataset consisted of 55 sequences each 669 bp in length with 214 polymorphic sites, 177 of which were parsimony informative. The ND5 dataset contained 58 sequences each 1046 bp in length with 304 variable sites, 227 of which were parsimony informative. The nuclear genes were far less variable: The H3 dataset contained 57 sequences with each 308 bp with 25 variable sites, 18 of which were parsimony informative. We analysed 58 sequences for the 18s gene fragment, which contained 567 bp, only one of which was variable and parsimony informative. The ITS2 dataset consisted of 54 sequences each with 364 bp with 11 variable sites, four of which were parsimony informative.

Our analyses revealed two well-defined groups contrasting with the current taxonomy (Fig. 2). One group consisted of the Palaearctic taxa, and the other one was composed of Nearctic taxa. Hence, the tribes Sphingonotini and Bryodemini appear to be polyphyletic groups. In both major clades, several subclades could be distinguished. In the Palaearctic clade, the deepest split corresponds to the established Sphingonotini and Bryodemini groups. In the Nearctic clade, the deepest split primarily describes a divergence event within the classically defined Sphingonotini genus *Trimerotropis*. This grouping is consistent with the groups A and B defined by White (1948, 1950, 1973) on the basis of chromosomal conformation. The Bryodemini genus *Circotettix* branches off within the chromosomal group B of *Trimerotropis* more recently. Hence, in the Neartic, this classically defined Bryodemini genus is a sister taxon to *Trimerotropis cyaneipennis* within a larger *Trimerotropis* clade.

The analysis of the concatenated dataset yielded a different topology. While the Nearctic clade still formed a monophylum (pp: 1.00), the two Palaeartic groups did not group together, but the Bryodemini formed the sister

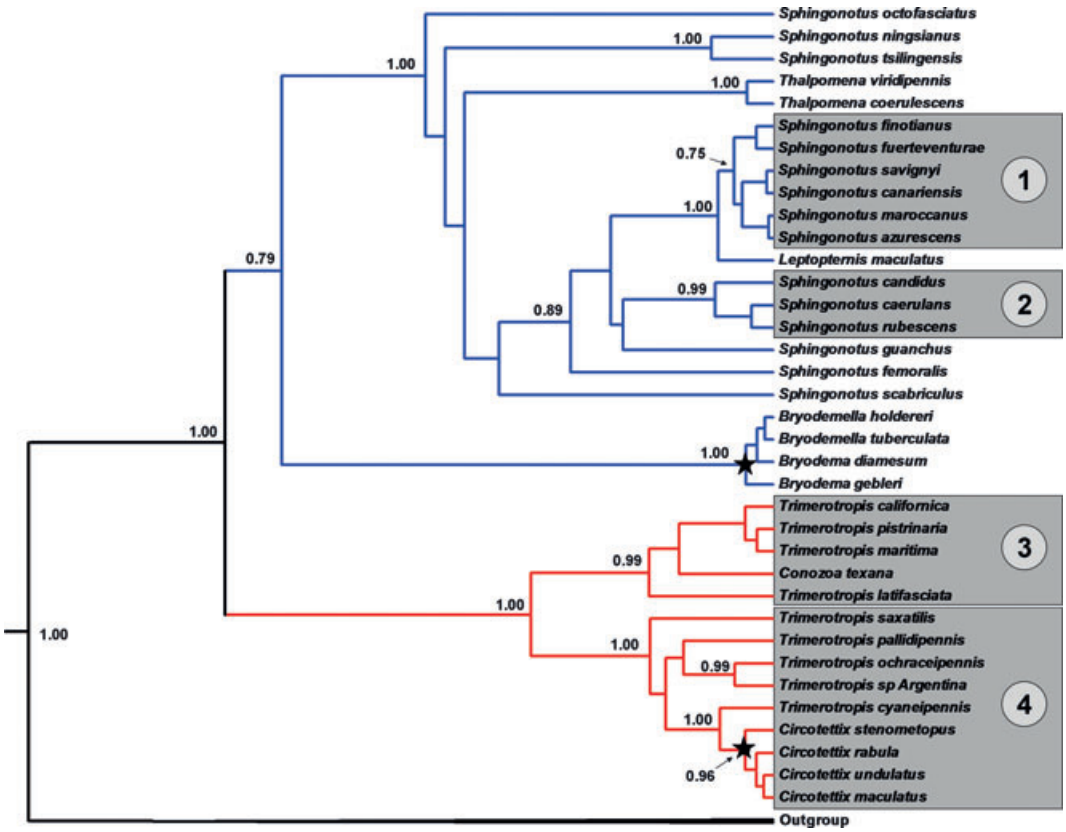

Fig. 2 Species tree generated with *BEAST analyzing all five sequenced loci simultaneously. Values at nodes represent posterior probabilities from Bayesian analyses (only posterior probabilities >0.70 are shown). Branch colors indicate geographic origin of species (red – New World, blue – Old World). Stars indicate the most parsimonious position for the evolution of broad wing morphology determined using ancestral state reconstruction with RASP (Maximum Parsimony). Numbers behind clades correspond to species groups identified in previous studies: (1) *Sphingonotus azurescens* group, (2) *Sphingonotus caerulans* group (Hochkirch & Husemann 2008), (3) *Trimerotropis* chromosomal group A, (4) *Trimerotropis* chromosomal group B (White 1948, 1950, 1973).

group to the Neartic clade (pp: 0.85), while the Palaearctic Sphingonotini formed a monophyletic group (pp: 0.90) at the basal position in the tree (Fig. 3). Our test of the two main hypotheses (common ancestry vs. convergence) and the additional hypothesis generated by the analysis of the concatenated dataset via Bayes factor analysis clearly supported hypotheses 1: convergence (Bayes factor 0.268), while both alternative hypotheses were rejected (Bayes factors H2: 363.804, H3: 89.878, Table 3).

Dating of the phylogeny yielded rough time estimates (Fig. 4). The molecular clock approach dated the split between Nearctic and Palaearctic lineages around 35 (±12, highest posterior density) Ma and placed the divergence of both lineages in the late Eocene or within the Oligocene. The radiation of American *Trimerotropis* grasshoppers started ca. 16 (±8) Ma in the Miocene with the split into the two major chromosomal groups A and B. The broad-winged clade (Nearctic Bryodemini) split off within the group B ca. 4 (±3) Ma. The divergence between the two major old world clades dated back much further and was estimated at around 32 (±13) Ma within the late Eocene or early Oligocene.

Maximum Parsimony reconstruction of ancestral wing morphology indicated that the broad winged phenotype evolved twice independently in the Palaearctic Bryodemini and in the Nearctic genus *Circotettix* (Fig. 2).

Discussion

Intercontinental relationships

Our analyses show that the current taxonomy does not reflect the phylogenetic relationships between the Nearctic and Palaearctic representatives of the tribes Bryodemini and Sphingonotini. Both tribes are polyphyletic, supporting the results of White (1973) and Fries *et al.* (2007) and stressing the need of a revision of the Oedipodinae tribes. In contrast to the current taxonomy our analyses indicate

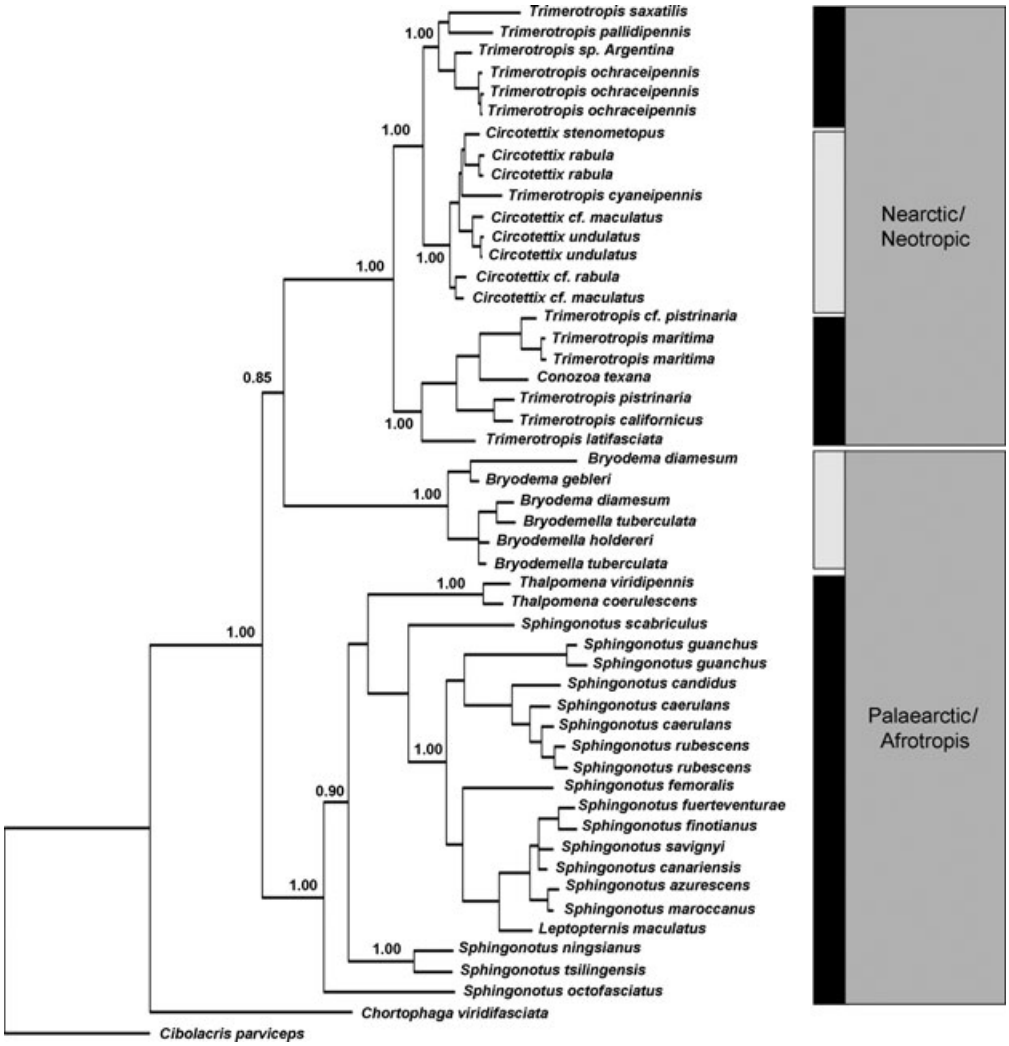

Fig. 3 Phylogenetic relationships as recovered from analysis of the concatenated dataset using MRBAYES; posterior probabilities are only show for main branches. Black bars indicate a narrow-winged morphology as typical for the Sphingonotini, while light grey represents the typical Bryodemini broad-winged morphology.

Table 3 Test of alternative hypotheses using Bayes Factor comparisons as implemented in TRACER v.1.5.0 following Marek & Bond (2006)

Hypothesis tested	Mean LnL ± SE	Bayes Factor	Phylogenetic inference
H_1: Geography	−14 256.20 ± 0.464	0.268	Supported
H_2: Taxonomy	−14 263.41 ± 0.499	363.804	Not supported
H_3: Concatenated data	−14 262.01 ± 0.464	89.878	Not supported

The null hypothesis (H_0) was generated using an unconstrained *BEAST analysis with four defined groups (Nearctic Bryodemini, Nearctic Sphingonotini, Palaearctic Bryodemini and Palaearctic Sphingonotini), the alternative hypotheses were generated using the same type of analyses but constraining the tree topology to the respective hypotheses: H_1: The four groups cluster by their geographical distribution (Fig. 1A), H_2: the groups cluster by their wing morphology and current taxonomic identity (Fig. 1B), H_3: the tree topology was constrained to reflect the intergroup relationships recovered in the concatenated analysis (Fig. 3). Harmonic mean of the unconstrained tree was LnL = −14 257.51 ± 0.477.

that two monophyletic lineages exist, one in the Palaearctic and one in the Nearctic. This suggests that only one dispersal event followed by a radiation led to the present distribution patterns. While the Palaearctic subclades correspond to the tribes they originally were assigned to, the two subclades recovered for Nearctic species do not. Based upon our molecular clock analysis, the split between the two Palaearctic tribes is much older than the split between the Nearctic lineages.

The two Nearctic subclades, clearly coincide with the groups A and B of *Trimerotropis* as defined by White (1948, 1950, 1973) based on the chromosomal arrangement. The group A, consisting of species of *Trimerotropis* and *Conozoa*, has only acrocentric chromosomes, while members of the group B, which includes some other

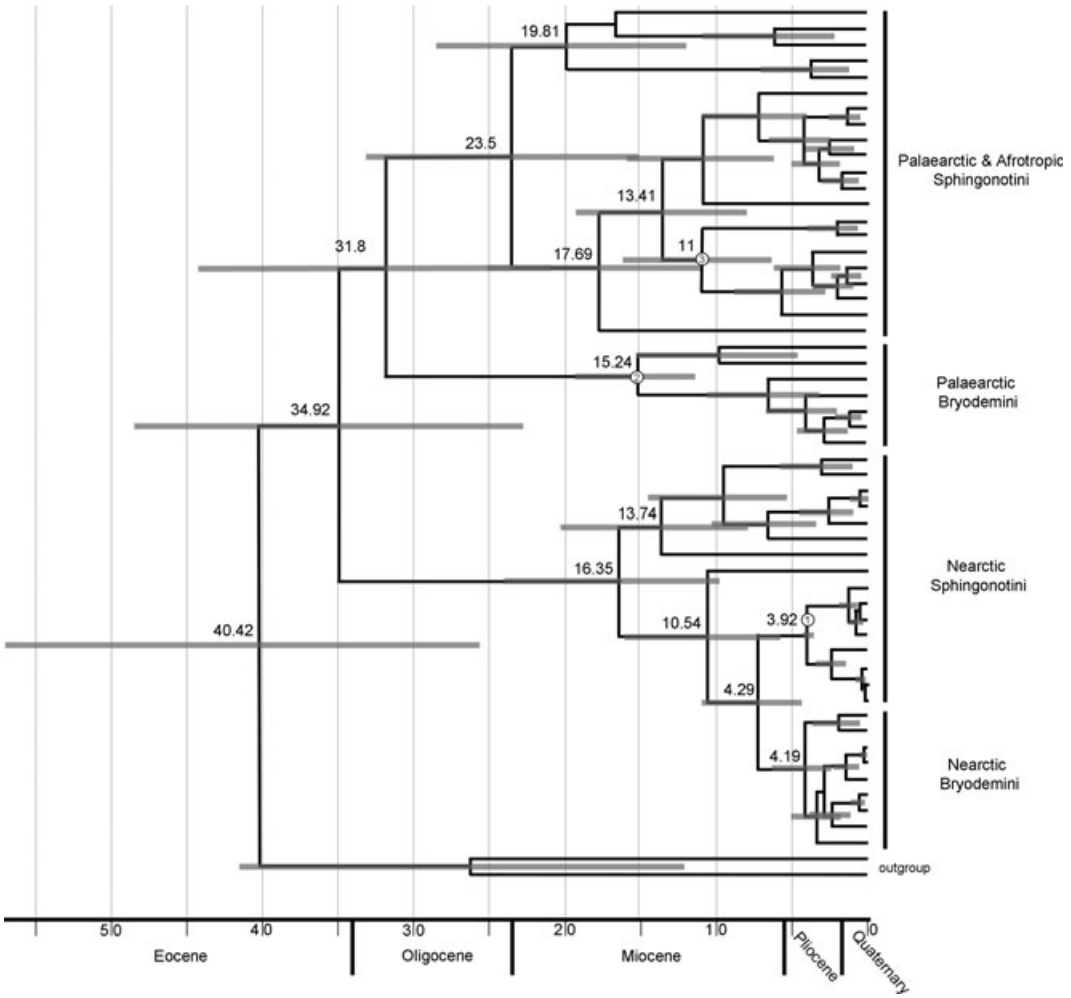

Fig. 4 Chronogram with 95% highest posterior density bars, based on BEAST analyses of the concatenated mtDNA dataset (1715 bp) with three calibration points; (1) fossil based (Bryodema croatica, lower Miocene), (2) *S. guanchus* (Gran Canary, max. age 15 Ma), and (3) Panama Isthmus, split of South American and North American lineages of *T. pallidipennis* (3.5–4 Ma); geological timescale (Gradstein & Ogg 2004) is shown below the chronogram.

Trimerotropis species and all *Circotettix* species, have metacentric chromosomes (White 1948). White (1973) had separated Palaearctic Bryodemini from *Circotettix*, because they strongly differ in their chromosomal makeup.

While the Palaearctic and Nearctic lineages are genetically distinct, the misclassified Nearctic genera share morphological and ecological features with Palaearctic taxa. Such morphological convergence is a common pattern in taxa living in similar environments and may be caused by 'ecological equivalence' (Shelford 1911). Uvarov (1977) already illustrated the morphological convergence of Caeliferan species with similar ecological niches belonging to different families or subfamilies. These results illustrate that outer morphological characters are often unreliable for drawing conclusions on the systematic relationships of taxa. Genetic data, such as DNA sequences and even the karyotypes used by White (1973), are more useful for defining higher taxonomic groups.

Convergent evolution of wing morphologies

The wings of Oedipodinae grasshoppers are likely to be under strong natural and sexual selection as they serve several functions. They are used in flight, communication (Husemann *et al.* 2011), thermoregulation (Gilman *et al.* 2008), camouflage (Cox & Cox 1974) and predator confusion (Kral 2010). It is thus not surprising that their use for drawing systematic conclusions is rather limited. By clearly rejecting the hypothesis of a common ancestry of both

phenotypes in the Oedipodines (Fig. 2), our data support the limited systematic and phylogenetic value of such characters.

The narrow winged phenotype appears to be the ancestral state, while the broad-winged morphology has evolved at least twice independently: in the Palaearctic Bryodemini and in the genus *Circotettix*, or got lost in the Neartic narrow winged clade as would be suggested by the concatenated analysis. The Palaearctic lineage (*Bryodema* and allies) is much older (one fossil is known from the lower Miocene) than the Nearctic lineage (*Circotettix*), whose origin has been dated at about 4 Ma. Interestingly, both groups are found in higher mountain ranges and hence are subjected to similar environmental conditions. Hence, the broad fore wings may confer selective advantage to Oedipodines living in high mountain ranges. However, some species of *Trimerotropis* also occur at higher elevations and do not show a broad winged morphology. This might be explained by a more recent invasion of this habitat. A formal test of this hypothesis is needed to conclusively infer the action of convergent adaptation to the habitat.

Vicariance vs. dispersal

Our analyses clearly support a single colonization event leading to the Neartic clade. This colonization event could have occurred either due to the break-up of Laurasia 90–65 Ma (Sanmartín *et al.* 2001) or through later dispersal. The molecular clock analyses placed the Nearctic/Palaearctic split at about 35 million years, which fails to support the hypotheses of Laurasian vicariance. Hence, the observed split among Nearctic and Palaearctic members of both tribes is probably the result of a dispersal event subsequent to the break-up of Laurasia. This is consistent with studies performed in a variety of other taxa with Holarctic distributions, where dispersal and subsequent diversification led to contemporary diversity patterns (Weider *et al.* 1999; Cook *et al.* 2004; Koehler *et al.* 2009; Vila *et al.* 2011). Considering the deeper splits within the Palaearctic lineage and the higher species diversity in the Palaearctic, it is most likely that colonization occurred from the Old to the New World, which is also supported by a more comprehensive study of band-winged grasshoppers which suggests this direction of colonization (Chapco & Contreras 2011). A more complete taxon sampling within the Oedipodinae is needed to fully test this.

Although we were able to assign the phylogeography of the taxa to a single dispersal event, the route of colonization cannot be determined with certainty. Our dating estimates reject the Thulean Land Bridge as colonization pathway, which existed between 55 and 50 Ma (McKenna 1983). The Beringian land bridge possibly represented a viable migration route for the ancestors of the present North American Oedipodinae. It was passable 55–35 Ma, 14–3.5 Ma and multiple times during the Pleistocene (Sanmartín *et al.* 2001), which is congruent with our dating estimates of this divergence event. Long-distance dispersal via flight appears to be unlikely considering the comparatively low flight ability of most taxa, but cannot be rejected. 'Floating islands' are another possible colonization mode that would not require a land bridge connection (Houle 1998).

Taxonomic conclusions

Based upon our results, we propose to keep the current tribe affiliation of the Palaearctic taxa. However, we assign the Nearctic genera *Trimerotropis*, *Conozoa*, *Circotettix* (as suggested in this study) as well as *Spharagemon* and *Dissosteira* (as suggested by Fries *et al.* 2007) into the purely Nearctic tribus Trimerotripi(ni) Blatchley, 1920. However, this is a provisional assignment until the systematic relationships among all North American Oedipodinae genera are resolved. Within the Nearctic clade, we found evidence for a clear split, which coincides with the chromosomal groups A and B, suggested by White (1948, 1950, 1973). These groups might be helpful for a higher classification according to tribes. Currently, these groups do not conform to the taxonomy. Particularly, monophyly of the genus *Trimerotropis* is rejected. We, therefore, synonymise *Circotettix* with *Trimerotropis*. *Conozoa texana* is assigned to the genus *Trimerotropis*. More comprehensive studies in the future might help to unravel the phylogenetic relationships within the genus *Trimerotropis* and possibly split up the genus as suggested by their chromosomal differences.

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