

Phylogeny of the dragonfly genus *Sympetrum* (Odonata: Libellulidae)

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Abstract The libellulid dragonfly genus *Sympetrum* has been recognized since 1833, but lacks any morphological synapomorphies to unite the taxon. Previous researchers have disagreed over which species belong in *Sympetrum*, bringing the monophyly of the genus into question. We use DNA sequence data from 6 genetic loci (16S, tRNA-valine, 12S, elongation factor 1 alpha, cytochrome oxidase subunit I, and the second internal transcribed spacer region) and 25 morphological characters (mainly genitalic) to test the monophyly of *Sympetrum* with Bayesian inference and maximum likelihood analyses. Under Bayesian inference, all *Sympetrum* species included in this study form a clade, which also contains the Hawaiian monotypic genus *Nesogonia*, often considered a close relative of *Sympetrum*. Phylogenetic analyses also reveal at least six strongly supported clades (treated as species groups) within *Sympetrum*, but relationships between these species groups remain unresolved or unsupported. Although the relationships between *Sympetrum* species groups remain unresolved, several species groups include taxa from multiple biogeographic regions/continents, and the species group sister to the rest of *Sympetrum* contains migratory species from the New World and Africa. This pattern suggests a complex biogeographic history in *Sympetrum* shaped by vicariance and dispersal. Preliminary estimates of the divergence dates

of *Sympetrum* species groups outline a rapid radiation of the groups approximately 32–38 million years ago, possibly influenced by cooling and drying climates of the late Eocene and early Oligocene.

Keywords Odonata · Anisoptera · Divergence dating · Libellulidae · Phylogeny · Rapid radiation

Introduction

Sympetrum Newman 1833 (Libellulidae) contains over 60 species and occurs on every continent except Australia (Needham et al. 2000; Walker and Corbet 1975). The genus has few tropical species (7 species endemic to Central and South America and 1 species in sub-Saharan Africa) and is most species-rich in the Holarctic (~13 species in North America and nearly 40 species in Europe or Asia). Most species of *Sympetrum* are predominantly red and are relatively small for dragonflies (most <40 mm long). *Sympetrum* species are found most often in habitats with slow or stationary water such as bogs, fens, or marshes where they are most typically observed perched on reeds or other vegetation.

The taxonomic limits of *Sympetrum* are problematic because the description of the genus appears to include no synapomorphies. The original generic description (Newman 1833) is vague at best. More recent re-descriptions (Needham et al. 2000; Walker and Corbet 1975) of the genus outline the taxon as a unique combination of non-unique characters. The most striking characteristic of the genus is an expanded,

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bilobed prothorax fringed with long setae; this character, however, is shared with several other libellulid genera such as *Celithemis* Hagen, *Erythemis* Hagen, *Erythrodiplax* Brauer, *Leucorrhinia* Brittinger, *Nesogonia* Kirby, and *Pachydiplax* Brauer (Walker and Corbet 1975). Some other characteristics of *Sympetrum* (e.g., slender legs, rounded head, slightly compressed pterothorax; Walker and Corbet 1975) are descriptive, but also are present in many other libellulid genera.

The lack of synapomorphies for *Sympetrum* could explain the long-standing debate over which taxa should be included in the genus. In 1936, Needham and Fisher erected *Tarnetrum* to contain two species previously placed in *Sympetrum*, *S. corruptum* (Hagen) 1861 and *S. illotum* (Hagen) 1861. *Tarnetrum* was successively expanded to include other species such as *S. nigrocreatum* (Calvert) and *S. gilvum* (Hagen) of South America (Borror 1945), *S. madidum* (Hagen) of western North America (Walker and Corbet 1975), *S. fonscolombii* (Selys), a migratory species from Africa (Schmidt 1987), and *S. roriamae* DeMarmels and *S. villosum* Ris, also of South America (Carle 1993). The status of *Tarnetrum* as a valid genus is not without controversy, as some authors later treated it as a subgenus of *Sympetrum* (Cannings 1981; Dunkle 2000; Needham et al. 2000; Walker and Corbet 1975) or considered it invalid (Gloyd and Wright 1959; Kormondy 1958, 1960; Paulson 2009). Further questions concerning the limits of *Sympetrum* involve the monotypic Hawaiian endemic *Nesogonia* Kirby, which also has been suggested as a possible sister to *Sympetrum* or may even belong within the genus (Carle 1993; Kiauta 1969).

The relationships within *Sympetrum* are even more unsettled than the validity of *Tarnetrum* or *Nesogonia*, because no phylogenetic work on *Sympetrum* has been done. Recent taxonomic work has focused on alpha taxonomy, with several new species described in the last 20 years (Cannings and Garrison 1991; Carle 1993; DeMarmels 1994, 2001; Han and Zhu 1997), and others invalidated (Pilgrim and von Dohlen 2007). North American species have been arranged into three 'sections' based on similarity of their genitalia (Walker and Corbet 1975), and some have been placed into a subgenus *Kalosympetrum* (Carle 1993). These North American groups are likely artificial, however, because the morphological similarities between Palearctic species and Nearctic species have never been addressed with respect to relationships. The Nearctic species have been regarded as a single lineage even though some North American species have greater morphological similarities to some Palearctic species than to the other North American groups. To date, there has been no phylogenetic analysis to test the monophyly of *Sympetrum* or to delineate species-groups based on clades instead of geographic range.

Apart from the taxonomic issues surrounding the genus, *Sympetrum* constitutes an interesting subject from a biogeographic perspective. The nearly global distribution of the genus could suggest that it is an ancient lineage whose distribution could be due to global vicariance events. Putative fossils of *Sympetrum*, however, are known only from a few species and from European specimens, and only as far back as the Upper Miocene (Gentilini 1988) when the positions of continents were very similar to the present. The presence of multiple fossil species from the Miocene would suggest that the genus is older, possibly dating to earlier periods when the positions of landmasses, and connections between them, were very different. Regardless of the age of *Sympetrum*, the historical biogeography of the genus is likely complex. The strong flight capabilities of *Sympetrum* species imply that dispersal could also play a major role in the current distribution of the genus. *Sympetrum* currently contains two migratory species (*S. corruptum* and *S. fonscolombii*), but the possibility that these two species belong in another genus (see above) complicates the potential role of migratory ancestors as dispersal agents for *Sympetrum*. Previous biogeographic work in *Calopteryx* damselflies, which are predominately Holarctic like *Sympetrum*, found that the genus arose in the Palearctic with the Nearctic taxa resulting from a single dispersal event to North America (Misof et al. 2000). The center of diversity for *Sympetrum* is the Palearctic, but the center of diversity for a lineage may not necessarily be the center of origin for that group (Lomolino et al. 2005). The morphological affinities between different groups of North American species and Eurasian species hint at the possibility of a complex biogeographic history with multiple dispersal events.

The goal of this study was to develop a robust phylogeny of *Sympetrum* to test the monophyly of the genus and address the historical biogeography of the group. Our taxon sample included 40 species, including the monotypic genus *Nesogonia*, and outgroup taxa from several related Libellulidae genera. Data were collected from morphological characters and DNA sequences of the nuclear genes elongation factor-1 α (EF1) and the second ribosomal internal transcribed spacer (ITS2), and the mitochondrial genes 16S/tRNA-val/12S, and cytochrome oxidase I (COI).

Materials and methods

Specimen collection, vouchers, and taxon selection

We sampled recently collected specimens from museum loan as well as freshly collected material. Fresh material was stored in acetone for approximately 24 h to remove

moisture and lipids. Specimens were then stored dry in polypropylene envelopes. Loan material included both acetone-dried specimens as well as specimens collected into 95–100 % ethanol. Voucher specimens were deposited into the Department of Biology Insect Collection, Utah State University, Logan, UT, or returned to the loaning institution or individual.

The 35 *Sympetrum* species included in the study represented all previously designated species groups and the full geographic range of the genus (Table 1). Two populations of *Sympetrum danae* were included because this widely distributed species actually may represent two cryptic species. The Hawaiian, monotypic genus *Nesogonia* was included as a possible synonym of *Sympetrum*, as well as five of the eight species formerly included in *Tarnetrum*; we follow Garrison et al. (2006) in regarding *Tarnetrum* as a junior synonym of *Sympetrum*. Three outgroup genera (*Celithemis*, *Leucorrhinia*, and *Rhyothemis*) were also represented: *Celithemis* and *Leucorrhinia* are the closest relatives of *Sympetrum*, and *Rhyothemis variegata* (Linnaeus) is a more distant outgroup (Pilgrim and von Dohlen 2008).

Morphological characters

Twenty-five morphological characters were coded for all *Sympetrum* taxa and outgroups *Celithemis* and *Leucorrhinia* (see Appendix for character descriptions and data matrix). All characters were treated as unordered and equally weighted. Although the entire morphology of these taxa was investigated, the most informative characters derived predominantly from male and female genitalia. Penises were dissected from ethanol-softened specimens for examination.

Molecular techniques

DNA was isolated from fresh specimens stored in 95 % ethanol or from relatively recent, dried museum specimens. To extract DNA, a middle and hind leg of each specimen was removed, leaving the remainder of the specimen as voucher. DNA was extracted with the High Pure PCR Template Preparation Kit (Roche Pharmaceuticals, Indianapolis, IN).

Sequence data were collected from the nuclear genes EF-1 α and ITS2, and the mitochondrial genes 16S, tRNA-valine, 12S, and COI. We also obtained sequences for the ITS1 region, but this locus was too variable to be aligned with confidence in homology; thus, we elected not to include it. ITS1 should be informative, however, for more focused analyses within species groups or closely related species. For EF-1 α , 16S, tRNA-valine, 12S, and COI, the PCR conditions, primers, and thermal cycler programs followed those described in Pilgrim and von Dohlen (2008).

For ITS2, the PCR conditions, primers, and thermal cycler programs followed those described in Pilgrim and von Dohlen (2007). PCR products were visualized on agarose gels stained with ethidium bromide, and successful amplifications were cleaned using standard isopropanol purification. PCR amplifications of the 16S through 12S gene of *Celithemis elisa* consistently failed, as did amplifications of the EF-1 α gene of *C. eponina*; therefore, the 16S/12S sequence of *C. eponina* and the EF-1 α sequence of *C. elisa* were pooled for the combined genetic analysis and are hereafter referred to as *Celithemis* sp.

DNA sequencing reactions were performed using either the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) or the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocols. Purified products were analyzed with either an ABI Prism 377 Genetic Analyzer or ABI 3730xl DNA Analyzer. All sequence data have been deposited in GenBank (see Table 1 for accession numbers).

All PCR products were sequenced in both directions and were assembled into complete contigs using Sequencher v4.1–4.8 (Gene Code Corp., Ann Arbor, MI). COI and EF-1 α data sets were aligned in Sequencher. Introns in the EF-1 α data set were removed prior to alignment. The 16S-tRNA-valine-12S and ITS2 data sets each were aligned in MUSCLE (Edgar 2004) with minor editing post-alignment. Most indels in the alignments were only 1–2 bp in length, and we did not remove any portions of the data sets because of unreliable alignment.

Phylogenetic analyses

For model-based inference, the appropriate models of evolution for individual genes were determined with MrModeltest v2.2 (Nylander 2004) (GTR + I + G in all cases). Model-based analyses were performed on the combined-gene data set. Bayesian inference (BI) was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Data were structured into six partitions: 16S + tRNA-val + 12S, EF-1 α codon positions 1 + 2, EF-1 α codon position 3, COI positions 1 + 2, COI position 3, and ITS2 (six partitions). The partition containing EF-1 α codon positions 1 + 2 was modeled as nst=2 and nucleotide frequencies as equal; all other partitions were modeled as nst=6 and base frequencies estimated. Analyses included two independent runs with three heated and one cold chain in each run. Trees were sampled every 1,000 generations for 10 million generations. Convergence, ESS values, and burn-in were assessed with Tracer v1.5 (Rambaut and Drummond 2007). Maximum-likelihood (ML) analyses were performed with Garli v2.0 at molecularevolution.org. The combined-gene data set was analyzed with partitions

Table 1 Specimen collection data

Species	Authority	Locality	Accession Numbers			
			EF-1	ITS2	16S/12S	COI
<i>Nesogonia blackburni</i>	McLachlan 1883	Ewa County, Oahu, Hawai'i	EF640508	n/a	EF640429	JQ772571
<i>Sympetrum ambiguum</i> †	Rambur 1842	Cape May Co., New Jersey, USA	JQ772620	EF636418	JQ772594	EF636300
<i>S. baccha</i>	Selys 1884	Ono City, Hyogo Pref., Japan	JQ772621	JQ772547	JQ772595	JQ772572
<i>S. corruptum</i> *	Hagen 1861	Box Elder Co., Utah, USA	EF640518	JQ772548	EF640439	JQ772573
<i>S. costiferum</i>	Hagen 1861	Marinette Co., Wisconsin, USA	JQ772622	EF636369	JQ772596	EF636249
<i>S. croceolum</i>	Selys 1883	Sonobe-cho, Kyoto Pref., Japan	JQ772623	JQ772549	JQ772597	JQ772574
<i>S. danae</i> (Eurasian-Beringia)	Sulzer 1776	Fairbanks-North Star Borough, Alaska, USA	JQ772624	EU243916	JQ772598	EU243816
<i>S. danae</i> (Nearctic)	Sulzer 1776	Wasco Co., Oregon, USA	JQ772625	EU243970	JQ772599	EU243886
<i>S. darwinianum</i>	Selys 1883	Ono City, Hyogo Pref., Japan	n/a	JQ772550	JQ772600	JQ772575
<i>S. depressiusculum</i>	Selys 1841	De Plateux, Noord-Brabant Prov., The Netherlands	JQ772626	JQ772551	JQ772601	JQ772576
<i>S. eroticum</i>	Selys 1883	Ono City, Hyogo Pref., Japan	JQ772627	JQ772552	JQ772602	JQ772577
<i>S. flaveolum</i>	Linnaeus 1758	Noordwijk, Zuid-Holland Prov., The Netherlands	JQ772628	JQ772553	JQ772603	JQ772578
<i>S. fonscolombii</i> *	Selys 1840	Fthiotida, Elada Sterea, Greece	EF640519	JQ772554	EF640440	JQ772579
<i>S. gracile</i>	Oguma 1915	Ono City, Hyogo Pref., Japan	JQ772629	JQ772555	JQ772604	JQ772580
<i>S. illotum</i> *	Hagen 1861	Clatsop Co., Oregon, USA	EF640520	JQ772556	EF640441	JQ772581
<i>S. infuscatum</i>	Selys 1883	Ono City, Hyogo Pref., Japan	EF640521	JQ772557	EF640442	JQ772582
<i>S. internum</i> †	Montgomery 1943	Fairbanks-North Star Borough, Alaska, USA	JQ772630	EF636428	JQ772605	JQ772583
<i>S. kunckeli</i>	Selys 1884	Ono City, Hyogo Pref., Japan	JQ772631	JQ772558	JQ772606	JQ772584
<i>S. maculatum</i>	Oguma 1922	Tojo-cho, Hyogo Pref., Japan	JQ772632	JQ772559	JQ772607	JQ772585
<i>S. madidum</i> *	Hagen 1861	Josephine Co., Oregon, USA	JQ772633	JQ772560	JQ772608	JQ772586
<i>S. meridionale</i>	Selys 1841	Koycegiz, Mudga, Turkey	JQ772634	JQ772561	JQ772609	JQ772587
<i>S. obtrusum</i> †	Hagen 1867	Bay Co., Michigan, USA	EF640522	EF636436	EF640443	EF636326
<i>S. pallipes</i> †	Hagen 1874	Cache Co., Utah, USA	JQ772635	EF636419	JQ772610	EF636329
<i>S. parvulum</i>	Bartenev 1913	Hiyoshi-cho, Kyoto Pref., Japan	JQ772636	JQ772562	JQ772611	JQ772588
<i>S. pedemontanum</i>	Allioni 1766	De Plateux, Noord-Brabant Prov., The Netherlands	EF640523	JQ772563	EF640444	JQ772589
<i>S. risi</i>	Bartenev 1914	Ono City, Hyogo Pref., Japan	JQ772637	JQ772564	JQ772612	JQ772590
<i>S. rubicundulum</i> †	Say 1839	Greene Co., Ohio, USA	JQ772638	EF636450	JQ772613	EF636330
<i>S. sanguineum</i>	Müller 1764	Noordwijk, Zuid-Holland Prov., The Netherlands	EF640524	JQ772565	EF640445	EF636237
<i>S. semicinctum</i>	Say 1839	Greene Co., Ohio, USA	EF640525	EF636400	EF640446	EF636276
<i>S. signiferum</i>	Cannings & Garrison 1991	Cochise Co., Arizona, USA	JQ772639	EF636414	JQ772614	EF636287
<i>S. speciosum</i>	Oguma 1915	Hyogo Pref., Japan	EF640526	JQ772566	EF640447	JQ772591
<i>S. striolatum</i>	Charpentier 1840	Noordwijk, Zuid-Holland Prov., The Netherlands	JQ772640	EF636362	JQ772615	EF636239
<i>S. uniforme</i>	Selys 1883	Ono City, Hyogo Pref., Japan	JQ772641	JQ772567	JQ772616	JQ772592
<i>S. vicinum</i>	Hagen 1861	Chippewa Co., Michigan, USA	JQ772642	JQ772568	JQ772617	EF636293
<i>S. villosum</i> *	Ris 1911	Puerto Montt, Chile	JQ772643	JQ772569	JQ772618	JQ772593
<i>S. vulgatum</i>	Linnaeus 1758	Grootmeer, Brabant Prov., The Netherlands	JQ772644	EF636367	JQ772619	EF636247
OUTGROUPS						
<i>Celithemis elisa</i>	Hagen 1861	Greene Co., Ohio, USA	EF640471	AF549600	n/a	n/a
<i>C. eponina</i>	Drury 1773	Dearborn Co., Indiana, USA	n/a	AF549603	EF640393	n/a
<i>Leucorrhinia glacialis</i>	Hagen 1890	Somerset Co., Maine, USA	EF640472	AF549573	EF640394	JQ772570
<i>L. hudsonica</i>	Selys 1850	Somerset Co., Maine, USA	EF640473	AF549580	EF640395	n/a
<i>L. intacta</i>	Hagen 1861	Summit Co., Ohio, USA	EF640474	AF549586	EF640396	n/a
<i>L. proxima</i>	Calvert 1890	Somerset Co., Maine, USA	EF640475	AF549588	EF640397	n/a
<i>Rhyothemis variegata</i>	Linnaeus 1763	Karnataka, India	EF640530	n/a	EF640451	n/a

*Species that have been included in the disputed genus *Tarnetrum* by various authorities

†Species placed in the subgenus *Kalosympetrum* by Carle (1993)

modeled as in strategy (ii) above, except with no invariant rate category; base frequencies and proportion of invariant sites were estimated. Two analyses of 500 bootstrap replicates were performed.

Analysis of combined molecular and morphological data sets was performed under BI. Morphological data were treated as a seventh, unlinked data partition with the model set to the standard Markov k model used for morphological characters in MrBayes.

Clock-constrained Bayesian inference and divergence time estimation

Clock-constrained phylogenetics and nodal age estimates on the combined-gene data set were performed with BEAST v1.6.2 (Drummond and Rambaut 2007), after preparing the input xml file with BEAUti v1.6.2 (in the BEAST package). Data were partitioned as in strategy (ii) above. Substitution models were set to GTR + G [following the recommendation of Stamatakis (2006) to omit the invariant category] for the mitochondrial RNA and nuclear ITS2 partitions. Models for protein-coding COI and EF-1 α were set to SRD06 (Shapiro et al. 2006), which partitions the data into 1st+2nd vs. 3rd codon positions and applies the HKY model with rate variation. All model parameters were unlinked across partitions, the clock model set to uncorrelated lognormal, and tree prior set to Yule process. Putative fossils of *Sympetrum* have been described from Late Miocene deposits (Gentilini 1988). However, there are two issues regarding their utility as calibrations for the dating analysis. First, in our opinion, these fossils (consisting solely of wings) are only dubiously assigned to *Sympetrum*. While they are definitely Libellulidae, we strongly suspect they are not *Sympetrum*. Second, they bear no affinity to any particular extant *Sympetrum* species, and thus cannot be assigned to an internal node in the phylogeny. At best, they place an absolute minimum date for the origin of the genus, which is almost certainly much older. Therefore, to calibrate the molecular clock, we used a divergence date estimated in Ware et al. (2008). These authors dated a phylogeny of Libelluloidea from mitochondrial and nuclear rRNA data, calibrated with multiple fossils applied to family-level and older nodes. The age of the node subtending *Sympetrum*, *Leucorrhinia*, and *Celithemis* was estimated at 65 Mya; thus, we set the treeModel.rootHeight in our analysis to a mean of 65.0 and standard deviation of 2.0. Priors were left at default values except for .mu priors and yule.birthRate, which were set to normal distributions based on results from preliminary runs. Two final runs of 50 million generations were performed, with auto-optimize operators enabled and trees and parameters

sampled every 10,000 generations. In addition, the analysis was run twice from an empty alignment to assess the influence of the priors on the posterior distribution. Tree files were combined with LogCombiner 1.6.2, and the consensus tree was calculated with TreeAnnotator 1.6.2 (both programs included in the BEAST package).

Results

Phylogenetic analyses

As the 16S, tRNA-val, and 12S loci were all amplified and sequenced together, these loci were also aligned together to produce an alignment of 1,680 characters with 483 variable sites. The COI alignment consisted of 752 bases with 254 variable sites. For nuclear loci, the EF-1 α alignment of 705 bp had 78 variable characters, and the ITS2 alignment of 383 bp had 153 variable characters.

BI and ML analyses of the genetic data set recovered similar topologies (Fig. 1) showing a monophyletic *Sympetrum* with six strongly supported clades (PP>95 %), each containing four to eight species (Fig. 1). We have designated each of these clades as species groups named for the most senior species in each group: *danae* group, *fonscolombii* group, *flaveolum* group, *infuscatum* group, *pedemontanum* group, and *vulgatum* group (Table 2). Our designated *fonscolombii* group includes species sometimes placed in the genus *Tarnetrum*, but does not include all the *Sympetrum* species that have been attributed to *Tarnetrum*, such as *S. illotum* and *S. madidum*. Bayesian and ML analyses lacked either support or resolution for relationships among the *danae*, *flaveolum*, *infuscatum*, *pedemontanum*, and *vulgatum* species groups (Fig. 1). These model-based analyses nested *Nesogonia* within *Sympetrum* (between the *fonscolombii* group and the rest of *Sympetrum*) with high posterior probability. BI analysis of the combined molecular and morphological data set produced an identical topology and similar PPs as with genetic data alone.

Divergence estimates and historical biogeography

Relaxed-clock BI produced a consensus topology with fully resolved relationships between species groups, although PP values for these relationships were below 0.9 (Fig. 2). This analysis also placed *Nesogonia* as sister to *Sympetrum* (but without support). Divergence time estimates suggest that *Sympetrum* arose nearly 50 million years ago (MYA), with the *fonscolombii* group being the oldest species group in the genus. These date estimates also indicate that the remaining five *Sympetrum* species groups arose ~30 to 40 MYA, with the ancestor of the *infuscatum* group diverging ~37.8 MYA,

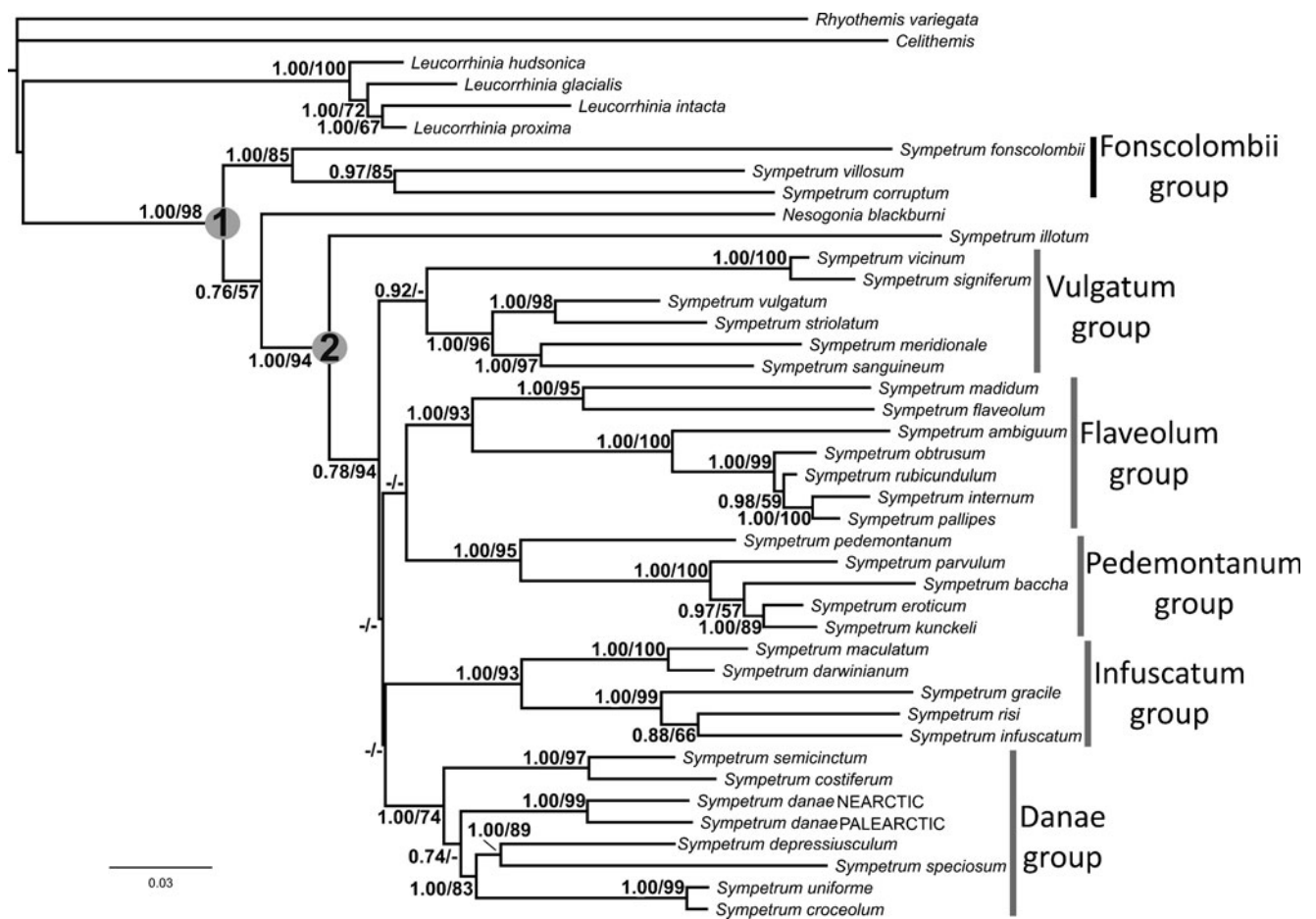


Fig. 1 Sample post-burnin tree from Bayesian analysis of the combined-gene (EF-1 α , ITS2, 16S, tRNA-valine, 12S, COI) data set. Numbers preceding the nodes represent BI posterior probabilities and

ML bootstraps, respectively. Unsupported or unresolved nodes are marked with a *hyphen*. Gray circle 1 denotes *Sympetrum sensu lato*. Gray circle 2 denotes *Sympetrum sensu stricto*

the *flaveolum* group diverging ~34.9 MYA, the *danae* group diverging ~32.9 MYA, and the *pedemontanum* and *vulgatum* groups diverging ~31.8 MYA.

The lack of well-supported relationships between species groups presents some difficulty for inferring the historical biogeography of *Sympetrum*, but the strongly supported

Table 2 *Sympetrum* species groups as supported by phylogenetic analysis

<i>S. fonscolombii</i> group	<i>S. pedemontanum</i> group	<i>S. infuscatum</i> group	<i>S. vulgatum</i> group
<i>S. corruptum</i>	<i>S. baccha</i>	<i>S. darwinianum</i>	<i>S. meridionale</i>
<i>S. fonscolombii</i>	<i>S. eroticum</i>	<i>S. gracile</i>	<i>S. sanguineum</i>
<i>S. villosum</i>	<i>S. kunkeli</i>	<i>S. infuscatum</i>	<i>S. signiferum*</i>
	<i>S. parvulum</i>	<i>S. maculatum</i>	<i>S. striolatum</i>
	<i>S. pedemontanum</i>	<i>S. risi</i>	<i>S. vicinum*</i>
			<i>S. vulgatum</i>
<i>S. danae</i> group	<i>S. flaveolum</i> group	Unclear Association	
<i>S. costiferum</i>	<i>S. ambiguum</i>	<i>Nesogonia blackburni</i>	
<i>S. croceolum</i>	<i>S. flaveolum</i>	<i>S. illotum</i>	
<i>S. danae</i>	<i>S. internum</i>		
<i>S. depressiusculum</i>	<i>S. madidum</i>		
<i>S. semicinatum</i>	<i>S. obtrusum</i>		
<i>S. speciosum</i>	<i>S. pallipes</i>		
<i>S. uniforme</i>	<i>S. rubicundulum</i>		

*Placement in this group is tentative due to support only in the Bayesian analyses

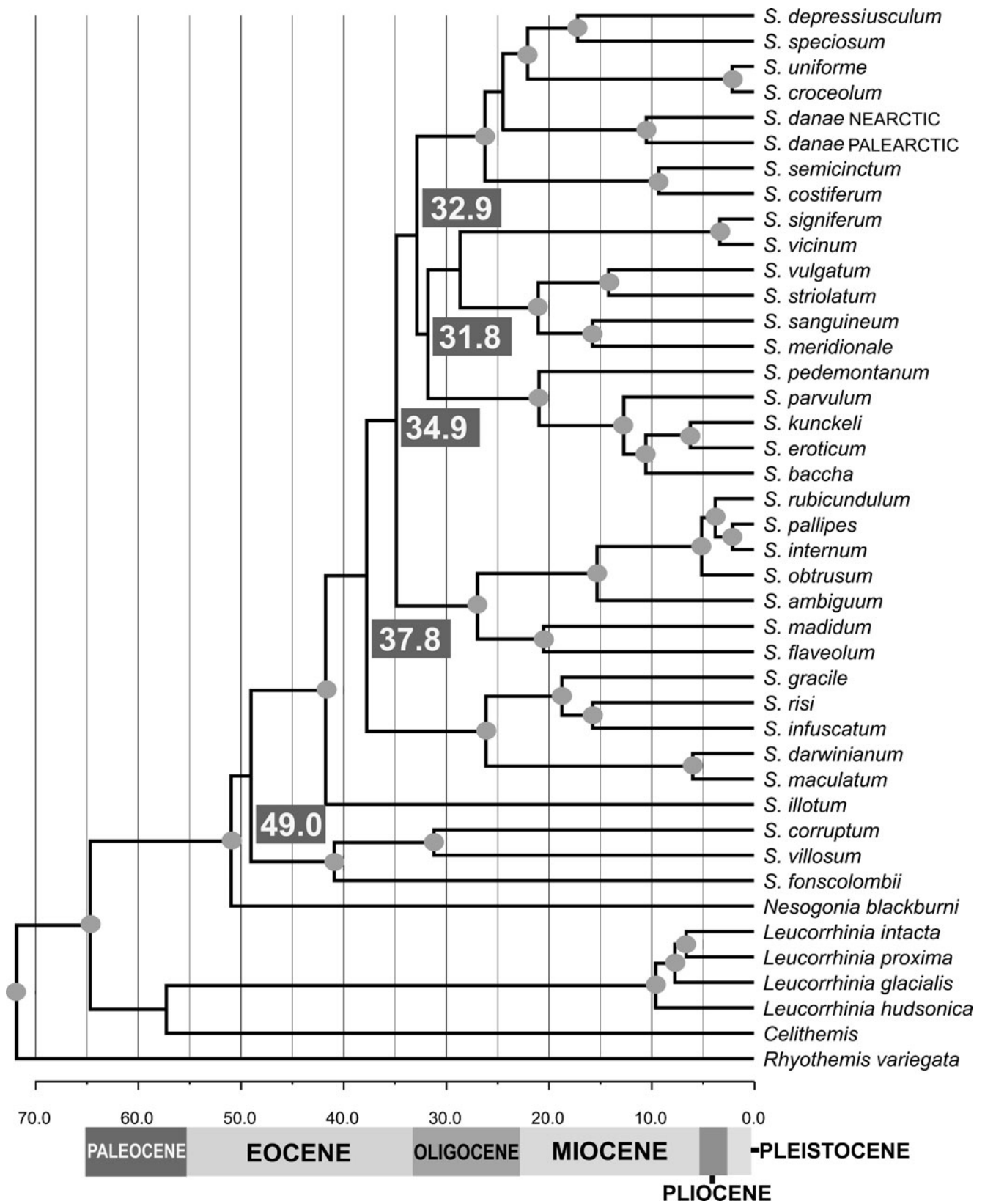


Fig. 2 Chronogram from the Bayesian relaxed-clock analysis of the combined-gene data set in BEAST. *Gray circles* denote nodes with 0.90 – 1.0 PP. *Gray boxes* show estimated divergence dates (MYA) for ancestors of species groups defined in this study

species groups and the geographic ranges of their extant taxa provide some information. The *fonscolombii* group has extant taxa that are migratory (*S. corruptum* and *S. fonscolombii*) and that cover a broad geographic range (*S. corruptum*: most of North America through Central America into northern South America; *S. villosum*: much of South America; *S. fonscolombii*: most of Africa and Europe). The *infuscatum* and *pedemontanum* groups have Palearctic taxa only, but the *danae*, *flaveolum*, and *vulgatum* groups have Nearctic and Palearctic extant species. Within the *danae* group, *S. costiferum* and *S. semicinctum* are exclusively Nearctic, *S. croceolum*, *S. depressiusculum*, *S. speciosum*, and *S. uniforme* are exclusively Palearctic, and *S. danae* encompasses a Nearctic lineage and a Eurasian-Beringian lineage. The *vulgatum* group contains Palearctic-restricted taxa such as *S. meridionale*, *S. sanguineum*, *S. striolatum*, and *S. vulgatum*, but if the Nearctic species *S. signiferum* and *S. vicinum* are included in this species group (strongly supported in BI), then the species group would be considered to have a Holarctic distribution. In the *flaveolum* group, only *S. flaveolum* has a Palearctic distribution, while the rest of the taxa in the species group are entirely Nearctic. None of the analyses show the Nearctic taxa as a single clade or the Palearctic taxa as a single clade.

Discussion

Our analyses provide strong evidence for circumscription of *Sympetrum* as a monophyletic taxon, although delineating exactly which species should be placed within the genus could require more study. Conforming to the ideal of classification based on robust monophyletic groups, two possible options emerge from our study. Option one would encompass all species currently considered as *Sympetrum*, plus *Nesogonia* and all species previously placed in *Tarnetrum* (e.g., Needham and Fisher 1936; Schmidt 1987; Carle 1993) (Fig. 1). Option two would restrict *Sympetrum* to the well-supported lineage of *S. illotum* and its sister clade of five species groups (Fig. 1). This option would preserve *Nesogonia* and reinstate *Tarnetrum* as represented by the *fonscolombii* group. We think it is premature to choose between these options at this time, because we were unable to acquire specimens of the other, rare South American *Sympetrum* species that could belong to the *fonscolombii* group. Until such material can be included in a future analysis, we suggest referring to the clade in option one as *Sympetrum sensu lato* and the clade in option two as *Sympetrum sensu stricto*. Our results do show, however, that if *Tarnetrum* is considered a valid genus in the future, *S. illotum* and *S. madidum* should

not be included because they did not cluster with the *fonscolombii* species group members formerly assigned to that genus. Our results also show that the subgenus *Kalosympetrum* (see Table 1) (Carle 1993) does constitute a clade, but is nested within the *flaveolum* species group, and therefore its utility as a taxon is reduced as it is now a subgenus nested within a species group. Future molecular studies should be combined with more extensive morphological studies to describe synapomorphies (if they exist) to support the choice of option one or two, above.

Lack of resolution and support along the backbone of the tree topologies suggest that that *Sympetrum* may have undergone a rapid radiation during the divergence of its species groups. All analyses produced topologies with short internode branches with low PP support between highly supported species group; such short internode branch lengths have been suggested as a hallmark of a rapid radiation (e.g., Whitfield and Kjer 2008). An argument could be made that the genetic loci chosen in this work simply lack the information necessary to resolve species group relationships; however, the nuclear and mitochondrial genetic loci used herein produced over 900 variable characters for determining relationships between ~40 taxa, strongly suggesting that locus choice likely has not hindered generation of a resolved phylogeny. Our divergence time estimates corroborate a period of rapid radiation in *Sympetrum*, indicating that five of the six species groups arose within a narrow interval of approximately 6 million years, as compared to the 50 million year history of the entire group (see below).

The species groups recovered in our study are supported by various morphological characters. Taxa within each species group have similar penis morphology (Fig. 3), such as a lack of large cornua (horn-like projections on the apical penis segment) in the *fonscolombii* group, flattened, down-curved cornua in the *flaveolum* group, and tusk-like, up-curved cornua in the *danae* and *vulgatum* groups. The *flaveolum* group is also united by the shapes of the male hamules and the female vulvar laminae (both are external accessory structures used in species recognition for mating). Outlining morphological characters to define *Sympetrum* species groups is beyond the scope of this work, but further research could test the use of genitalia characters for placing *Sympetrum* species not included herein to the proper species group.

Given the unresolved nature of the relationships between most of the *Sympetrum* species groups, the divergence estimates of the species groups should be considered preliminary. Unfortunately, the fossil record for *Sympetrum* (Gentilini 1988) is questionable, and the

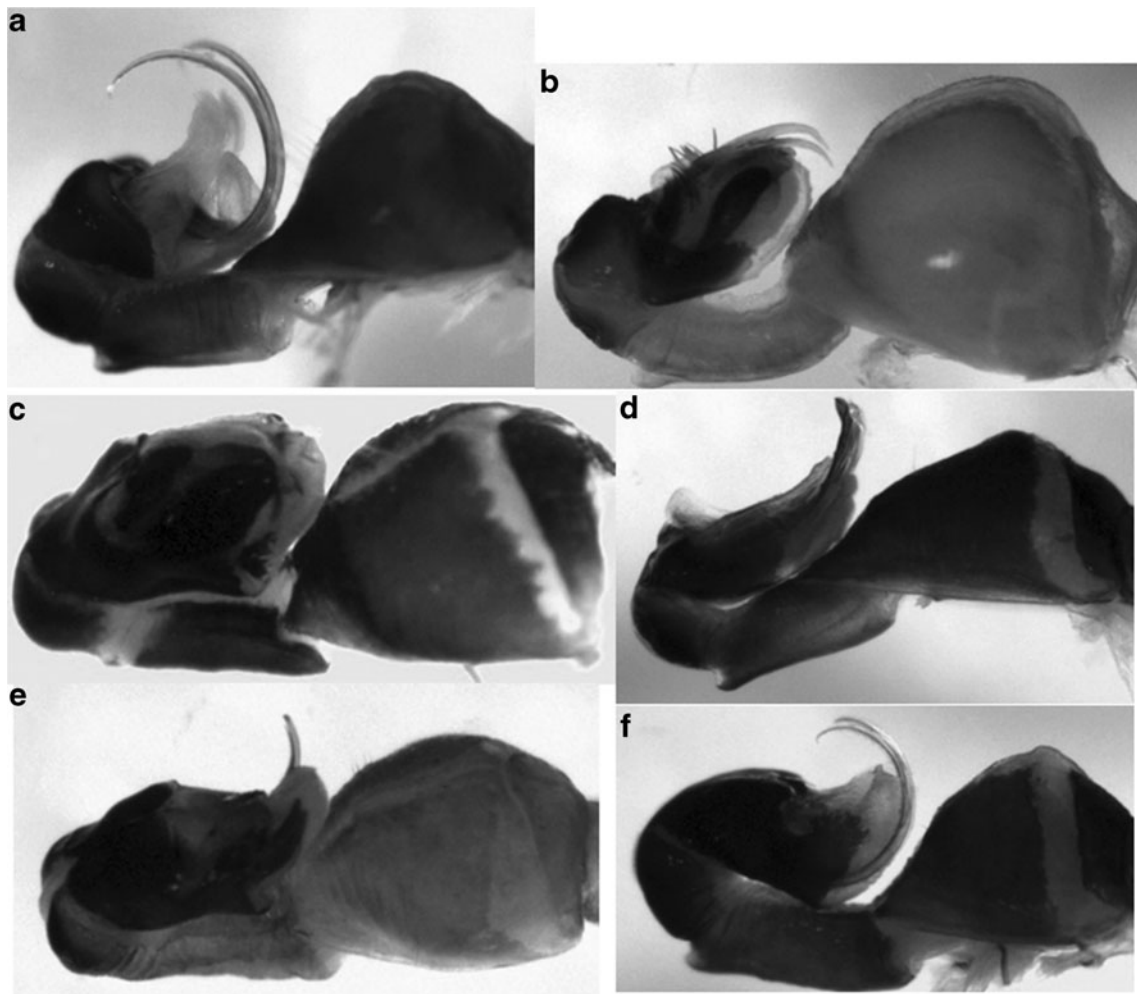


Fig. 3 Comparison of penis morphology across the *Sympetrum* species groups. **a** *Danae* group, *S. uniforme*; **b** *flaveolum* group, *S. rubicundulum*; **c** *fonscolombii* group, *S. corruptum*; **d** *infuscatum* group, *S. risi*; **e** *pedemontanum* group, *S. pedemontanum*; **f** *vulgatum* group, *S. striolatum*

use of a secondary calibration from another study was necessary (Ware et al. 2008). Those caveats being considered, however, the divergences between many of the *Sympetrum* species groups do coincide with known changes in global climate. Our results suggest five of the six species groups arose during the late Eocene to early Oligocene when the global climate became cooler and drier, and increases in the volume of polar ice caps led to drops in sea level (Sanmartin et al. 2001; Miller et al. 2008). These changes in climate could have fragmented ancestral *Sympetrum* populations leading to divergence and speciation.

Although this phylogenetic work did not recover highly supported relationships between many of the *Sympetrum* species groups, we may still draw several conclusions about the historical biogeography of this genus. Neither the Nearctic nor Palearctic *Sympetrum* species forms exclusive clades, and several of the species groups include taxa from

both regions. This implies that both dispersal and vicariance have shaped the biogeographic history of the genus, including several dispersal events between the Nearctic and Palearctic. Our divergence date estimates and tree topology also imply the importance of dispersal, as it places most cladogenesis in the last ~30 million years, which is well after major vicariance events induced by continental drift. This period in the late Tertiary, however, was marked by continued climate change, appearance, and submergence of Nearctic-Palaearctic land bridges, mid-continental orogeny, and erosion—all contributing to the possibility of multiple disruptions in gene flow, or facilitation of dispersal (Sanmartin et al. 2001; Denk et al. 2010). As the earliest branching lineages in the phylogeny, the *fonscolombii* group, *Nesogonia*, and *S. illotum* could provide insight into the geographic origins for *Sympetrum*. The current broad geographic ranges encompassed by these lineages (the *fonscolombii* group extends through North America,

Central America, South America, Europe, and Africa; *Nesogonia* occurs in Hawaii; *S. illotum* occurs in North and Central America), however, confound our efforts to place a geographic origin for *Sympetrum*. Their distributions could even suggest a point of origin in the Southern Hemisphere, well outside the current center of diversity for *Sympetrum* (Asia). Future systematic studies of *Sympetrum* should attempt to include the other South American species to help address the geographic origin of this genus.

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Appendix

Descriptions of the morphological characters used in the phylogenetic analyses.

1. Cornua of penis: (0) absent; (1) two cornua present; (2) four cornua present.

Cornua are structures that are believed to aid in the removal of sperm from previous matings. They do not occur on all male odonate genitalia, but are found in pairs when they do occur. All *Sympetrum* species had at least one pair of cornua. As the cornua were not found in the outgroup species, these taxa were coded as missing for characters 2, 3, 4, 9, 10, and 17.

2. Cornua curve: (0) anterior pointing; (1) posterior pointing.

Only members of the *flaveolum* group had posterior-directed cornua (Fig. 4a). All other clades of *Sympetrum* exhibited anterior pointing cornua (Fig. 4b, c).
3. Cornua thickness: (0) flattened; (1) thin/cylindrical.

The *S. flaveolum* and *S. infuscatum* clades had flattened cornua (Fig. 4a), along with *S. illotum* and *N. Blackburni*, whose affiliations to species-clades were unclear. The basal clade (*fonscolombii* group) had cylindrical, but shortened cornua. The other species clades (*danae*, *pedemontanum*, and *vulgatum* groups) all exhibited long cylindrical cornua (Fig. 4b, c). *Sympetrum signiferum*, a member of the *danae* group, had flattened cornua.
4. Cornua tips: (0) tapering smoothly to point; (1) hooked at point; (2) rounded tips.

The distal ends of the cornua tapered smoothly to a point for all the members of the *danae*, *flaveolum*, and *vulgatum* species groups. Members of the

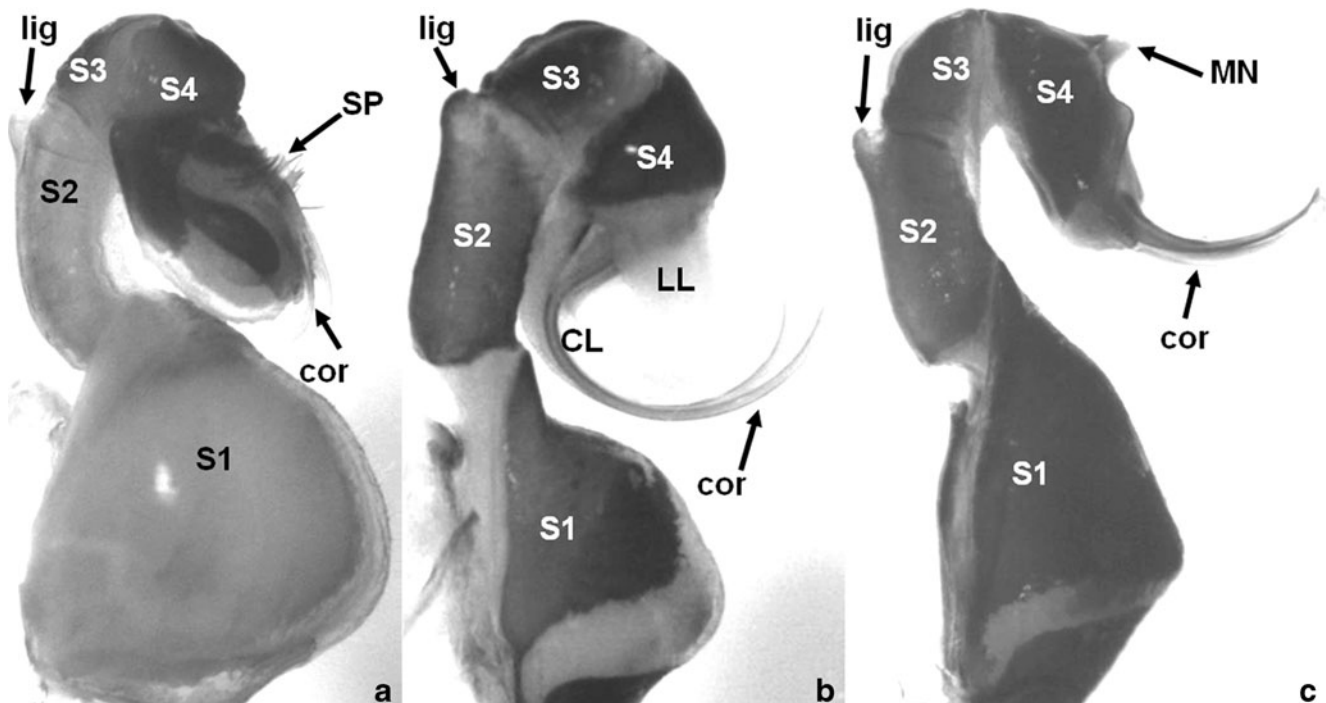


Fig. 4 Lateral views of representative *Sympetrum* male genitalia to identify coded morphological characters: **a** *S. rubicundulum*; **b** *S. danae*; **c** *S. gracile*. Labeled structures: *CL* central lobe; *cor* cornua;

LL lateral lobe; *lig* ligula notch; *MN* median nodule; *S1-S4* penis segments 1 to 4; *SP* setal patch

fonscolombii and *pedemontanum* clades along with *N. blackburni* had cornua that were distinctly hooked at their distal ends (Fig. 4). The exception for this second state was *S. pedemontanum*. *Sympetrum illotum*, only, had cornua that were rounded into paddle-like shapes at the tip.

5. Setal patches of S4 of penis: (0) absent; (1) present.
These tufts of wide setae (Fig. 4a) were found only in members of the *flaveolum* group, but not in *S. flaveolum* or *S. madidum*.
6. Basal medial nodule on S4 sclerite of penis: (0) absent; (1) present.
For most *Sympetrum*, the distal edge of the S4 sclerite was without ornamentation. The *flaveolum* and *infuscatum* species groups, however, had a distinct median nodule along this distal margin (Fig. 4). *Sympetrum sanguineum* of the *vulgatum* group also exhibited this nodule.
7. Width of penile S4: (0) widest at base; (1) widest apically where cornua originate; (2) subequal width throughout.
For *Sympetrum* species, the fourth segment of the penis was either widest at its base at the distal margin of the third segment, widest at its distal margin, or nearly the same width throughout its length. Members of the *pedemontanum* group were the only taxa to exhibit a wider base than distal margin for S4. The *flaveolum* and *vulgatum* groups along with *S. illotum* and *N. blackburni* had S4 sclerites that were widest at their distal margins. Members of the *danae*, *fonscolombii*, and *infuscatum* groups had S4 sclerites of nearly equal widths throughout. Within these clades, however, several species (*S. danae*, *S. darwinianum*, *S. depressiusculum*, *S. frequens*, and *S. maculatum*) had S4 sclerites that were widest at their distal margins. This character certainly varied in its utility for distinguishing species groups.
8. Ligula notch of S2 of penis: (0) prominent/hooked but less than 1/3 width of S2; (1) larger than 1/3 width of S2.
The ligula is a hooked structure responsible for helping hold the penis internally when not in use. All *Sympetrum* species, except *S. villosum*, had ligulae that were less than a third of the width of the second penis segment. The outgroup *Leucorrhinia* species had larger ligulae than those found in *Sympetrum*.
9. Cornua origin: (0) in plane of lateral lobes of S4; (1) above plane of lateral/central lobes of S4.
For most *Sympetrum* species, the cornua originated from the same area of S4 as the lateral lobes. For *N. blackburni*, *S. illotum*, and the *flaveolum* group, the

cornua arose from the S4 margin above where the lateral and central lobes originated.

10. Distance between cornua: (0) subparallel along length; (1) diverging apically; (2) converging apically.
For the *flaveolum* and *infuscatum* groups, and also for *S. illotum*, the cornua were basically parallel along their length. When viewed ventrally, the cornua of the *danae*, *fonscolombii*, *pedemontanum*, and *vulgatum* groups along with *N. blackburni* were distinctly divergent along their length. Converging cornua was an autapomorphy for *S. vicinum*.
11. Penis vesicle (S1): (0) smooth at base; (1) basal margin projecting outward.
12. Lateral margin of S1 of penis: (0) smoothly curved; (1) angular projection at widest point.

Characters 11 and 12 showed variability within the *Sympetrum* clades and therefore were not phylogenetically informative with respect to relationships among species groups. These characters could be useful as a key character, but not as a synapomorphy for a species group.

13. Lateral margin of S4 sclerite of penis: (0) weak/absent; (1) expanded ventrally beyond cornua; (2) expanded dorsally behind cornua; (3) expanded dorsally and ventrally beyond cornua.
The lateral margin of the S4 sclerite had multiple states. *Sympetrum gracile*, *S. infuscatum*, and *S. risi* had S4 sclerites that were not expanded on their lateral margins. Members of the *flaveolum*, *fonscolombii*, and *pedemontanum* groups had S4 sclerites that were expanded only ventrally beyond the cornua. The S4 sclerites were expanded dorsally for *N. blackburni*, *S. darwinianum*, *S. illotum*, *S. maculatum*, and the *danae* group. The *vulgatum* group had S4 sclerites that were expanded dorsally and ventrally, except for *S. signiferum* and *S. vicinum*, which had sclerites that were only ventrally expanded.
14. S4 sclerite of penis surface: (0) smooth; (1) heavily contoured with bumps and ridges.

A heavily contoured S4 sclerite was a synapomorphy for the *flaveolum* group and did not occur in any other taxa.

15. Central unexpanded lobe of S4 of penis: (0) in line w/ lateral lobes; (1) above plane of lateral lobes; (2) below plane of lateral lobes.

For many taxa, the lateral and central lobes of the last segment of the penis were small and arranged in the small plane (state 0) as in the *infuscatum* group. Some species of the *pedemontanum* and *vulgatum* groups had a central lobe that was above the plane of the lateral lobes (state 1).

Many taxa, including the outgroups *Celithemis* and *Leucorrhinia*, *S. illotum*, *N. blackburni*, and the *fonscolombii* and *danae* groups, had a central lobe that was well below the plane of the lateral lobes (state 2).

16. Width of S1 of penis: (0) more than $\frac{1}{2}$ S1 length; (1) less than $\frac{1}{2}$ S1 length.

For most species, including outgroups, the width of the 1st penial segment was much greater than half its length. *Sympetrum fonscolombii* and *S. villosum* of the *fonscolombii* group were the only species that had a thin 1st penial segment.

17. Visibility of cornua: (0) obscured by central/lateral lobes of S4; (1) visible along nearly entire length.

For species in the *fonscolombii* group, the cornua are so short that their visibility is obscured by the central and lateral lobes of the last penis segment. This character state was a synapomorphy for this species clade.

18. Ventral tooth of cerci: (0) prominent; (1) weak; (2) absent.

In many libellulid taxa, the cerci (superior anal appendages) of the male have a prominent tooth-like projection on the ventral edge prior to the cerci tapering to a point. This tooth was found in outgroups, and all species of the *flaveolum*, *infuscatum*, and *pedemontanum* groups. This tooth was completely absent in the *fonscolombii* group, but varied between weak and prominent within the *danae* and *vulgatum* groups.

19. Curve of dorsal side of cerci: (0) straight; (1) curved dorsally; (2) curved ventrally.

In libellulids, the cerci can remain uncurved along their length, or can curve dorsally or ventrally as they taper to a point. In this study, ventral curving was rare and only occurred in the outgroup taxa *Celithemis*, and *Leucorrhinia hudsonica*, and in the ingroup taxa *N. blackburni* and *S. illotum*. All the species of the *danae*, *fonscolombii*, *infuscatum*, and *vulgatum* groups had uncurved cerci. The species of the *pedemontanum* group consistently had dorsal curving cerci, and the *flaveolum* group varied between uncurved and dorsally curved cerci. The utility of this character may, however, be somewhat doubtful due to individual variation. As the adult emerges from the exuviae, the cerci may be distorted and would then harden, possibly with curving that may not occur for all members of that species.

20. Epiproct length: (0) subequal to ventral projection of cerci; (1) longer than the ventral projection of cerci; (2) shorter than the ventral projection of cerci.

In Anisoptera, the epiproct is the inferior anal

appendage of the male and is typically shorter than the full length of the cerci. The epiproct may extend beyond the ventral projection of the cerci (see character 19), be subequal in length to this projection, or not reach the projection. The *danae* and *fonscolombii* groups were consistent with all species having an epiproct subequal to the ventral projection of the cerci. The species of the *infuscatum* and *pedemontanum* groups had epiprocts that extended beyond the ventral projection. Members of the *flaveolum* and *vulgatum* groups were variable with some species having a subequal length, and some species having a much shorter epiproct.

21. Dorsal apical teeth of epiproct: (0) absent; (1) present.

The distal edge of the dorsum of the epiproct often had several tooth-shaped projections. After examining all the taxa here, only *Nesogonia blackburni* was found to be without these tooth-like projections.

22. Genital lobe shape: (0) pointed; (1) rounded; (2) quadrate.

The genital lobe of libellulids projects from the posterior margin of the genital fossa in males. This lobe may taper to a point, have a rounded margin, or have a quadrate shape. The species of the *danae* group all had rounded genital lobes. The only taxon to have a quadrate lobe was *S. corruptum*. The other species clades varied between pointed and rounded genital lobes. The utility of this character was doubtful, partly due to within-clade variation, but also because of difficulty coding individuals as either pointed or rounded.

23. Genital lobe angle: (0) ventral; (1) ventro-posterior; (2) posterior.

The genital lobe may point directly ventral, ventro-posterior, or directly posterior. All species of the *infuscatum* group had ventro-posterior angled genital lobes, but the other species clades varied in the states within these clades. Coding this character was difficult, because consistently determining the angle of the lobe was problematic. Intraspecific variation occurs for some species.

24. Carina of abdominal segment 4: (0) present; (1) absent.

The presence of a transverse carina on the 4th abdominal segment was used as a key character in Needham et al. (2000), and was described as a possible character for validating *Tarnetrum*. Further examination of this character showed that it may vary intraspecifically, and that it was found in many other taxa that were never considered to belong in *Tarnetrum*.

25. Lateral ridges of occiput: (0) absent; (1) present.

Lateral ridges were found only on species of the *flaveolum* group, except for *S. flaveolum* and *S. madidum*.

Morphological Character Data Matrix

Inapplicable data = -. Polymorphic characters [01] = A.

	1	1111111112	22222
	1234567890	1234567890	12345
<i>Celithemis</i> sp.	0---0010--	101021-021	10210
<i>Leucorrhinia glacialis</i>	0---0012--	201021-002	11-10
<i>Leucorrhinia hudsonica</i>	0---0012--	201021-022	11-10
<i>Leucorrhinia intacta</i>	0---0012--	201021-002	1--10
<i>Leucorrhinia proxima</i>	0---0012--	201021-002	11-10
<i>Nesogonia blackburni</i>	1101001011	1020211021	01110
<i>Sympetrum ambiguum</i>	1100011010	0011211011	10110
<i>Sympetrum baccha</i>	1011000001	1010011011	11200
<i>Sympetrum corruptum</i>	1011002001	0110210200	12100
<i>Sympetrum costiferum</i>	2010002001	1020211100	11100
<i>Sympetrum croceolum</i>	2010002001	0020211100	11200
<i>Sympetrum danae</i> *	2010001001	0020211000	11200
<i>Sympetrum darwinianum</i>	1001011000	0020011001	11110
<i>Sympetrum depressiusculum</i>	2010001001	1120211000	11110
<i>Sympetrum eroticum</i>	1011000001	1010111011	10000
<i>Sympetrum flaveolum</i>	1100011010	0111211000	11010
<i>Sympetrum fonscolombii</i>	1011002001	0010200200	11210
<i>Sympetrum gracile</i>	1001012000	1000011001	10100
<i>Sympetrum illotum</i>	1102001010	1120211120	10200
<i>Sympetrum infuscatum</i>	1001012000	1100011001	10100
<i>Sympetrum internum</i>	1100111010	10110110A1	10111
<i>Sympetrum kunckeli</i>	1011000001	1010111011	10100
<i>Sympetrum maculatum</i>	1001011000	1120011001	11100
<i>Sympetrum madidum</i>	1100011010	0011211000	11010
<i>Sympetrum meridionale</i>	1010001001	0130111001	10100
<i>Sympetrum obtrusum</i>	1100111010	0011011011	10111
<i>Sympetrum pallipes</i>	1100111010	0011011011	101A1
<i>Sympetrum parvulum</i>	1010000001	1010011011	10200
<i>Sympetrum pedemontanum</i>	2001001000	0010011010	11100
<i>Sympetrum risi</i>	1001012000	0000011001	11100
<i>Sympetrum rubicundulum</i>	1100111010	0011011011	10111
<i>Sympetrum sanguineum</i>	2010011001	0030111100	11100
<i>Sympetrum semicinctum</i>	2010002001	1020211100	11010
<i>Sympetrum signiferum</i>	1000001001	0010011001	11010
<i>Sympetrum speciosum</i>	2010002001	0120211100	11100
<i>Sympetrum striolatum</i>	1010001001	1030011200	111A0
<i>Sympetrum uniforme</i>	2010002001	0020211200	11200
<i>Sympetrum vicinum</i>	1010001002	1110011001	10000
<i>Sympetrum villosum</i>	1011002201	0010200200	10210
<i>Sympetrum vulgatum</i>	1010001001	1130111102	111A0

*character states used for both Nearctic and Palearctic lineages

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