INCOMPLETE BARRIERS TO HETEROSPECIFIC MATING AMONG *SOMATOCHLORA* SPECIES (ODONATA: CORDULIIDAE) AS REVEALED IN MULTI-GENE PHYLOGENIES

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ABSTRACT

Somatochlora, commonly known as the striped emeralds, is an enigmatic genus whose systematics have lagged other Odonata genera, with the last revision done by Walker (1925). North American *Somatochlora* inhabit fens, bogs, and forest streams, with most closely related species sharing a sympatric range. As a result, *Somatochlora* males have elaborate claspers which are species-specific and provide a morphological barrier to heterospecific mating, but exceptions have been observed. The objective of this project was to investigate the occurrence of heterospecific mating between North American *Somatochlora* species as inferred from multi-gene phylogenies. We employed the use of two mitochondrial genes (*COI* and *ND3*) and two nuclear genes (*EF1-α* and *ITS2*) to construct well-substantiated phylogenies using a maximum parsimony optimality criterion. Compared to nuclear genes (nDNA), mitochondrial genes (mtDNA) have a high nucleotide substitution rate, thereby allowing for the genetic discrimination of populations and species. Monophyly of mtDNA lineages is expected for closely related species because ancestral mtDNA lineages go extinct after a speciation event four times faster than nDNA lineages. Observation of non-monophyletic mtDNA lineages but monophyletic nDNA lineages between *Somatochlora* sister-species would indicate mtDNA introgression and suggest heterospecific matings. Our results highlighted three instances of heterospecific mating in the following groups: 1) *S. hineana* + *S. tenebrosa*; 2) *S. kennedyi* + *S. forcipata* + *S. franklini*; 3) *S. calverti* + *S. provocans* + *S. filosa*. In addition, the recovered topology accurately reflected previous taxonomic understanding of the genus. These multi-gene phylogenies of North American *Somatochlora* are the first, providing a foundation for future ecological and evolution studies and knowledge for effective decision-making and public policy, which is especially important for endangered species, *Somatochlora hineana*.

This thesis is dedicated to my family, friends, and the color brown.

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CHAPTER 1:
INTRODUCTION

Odonata (dragonflies and damselflies) have arisen as a unique ecological study group owing to their vagility, aquatic/terrestrial life history, unique sexual reproduction, and as bioindicators for a changing landscape of the Anthropocene (Bybee *et al.*, 2016). Odonata comprise one of the oldest winged insect lineages, and many phylogenetic studies at the ordinal, familial, generic, and specific level have been carried out (Misof *et al.*, 2014; Troast *et al.*, 2016; Bybee *et al.*, 2021). While Odonata systematics is developed, especially compared to other insect lineages, questions remain regarding basal relationships. Libelluloidea is a superfamily of dragonflies (infraorder Anisoptera) that is generally agreed to include families Synthemistidae, Macromiidae, Corduliidae, and Libellulidae (Carle *et al.*, 2015). Previous studies have struggled to find monophyly of Corduliidae, with some showing the family is paraphyletic (Blanke *et al.*, 2013) and a lack of resolution of the intra-familial relationships of Corduliidae (Carle *et al.*, 2015; Bybee *et al.*, 2021). A foundational systematic understanding of Odonata is essential for providing a framework for other causal branches of biology (*e.g.*, conservation), especially for Corduliidae which has the greatest Species-of-Greatest-Conservation-Need among Odonata families (Bried and Mazzacano, 2010). *Somatochlora* dragonflies, commonly known as the striped emeralds, are an enigmatic group of odonates characterized by green compound eyes and pale thoracic markings. Their bodies are covered in dark metallic browns, greens, blues, and blacks, while their thoraxes have yellow spots or stripes. *Somatochlora* species are morphologically similar and often difficult to identify (Mills, 2015). Species identification involves analyzing the shape and position of claspers and genital plates. *Somatochlora* is the largest genus in the family Corduliidae; however, the systematic understanding of *Somatochlora* has lagged behind other Odonata genera primarily due to their remote haunts and unique ecological requirements (Walker, 1925; Mead, 2021). Previous phylogenetic studies involving *Somatochlora* have used mitochondrial DNA (Kohli *et al.*, 2018; Walker *et al.*, 2020) or genomic data (Bybee *et al.*, 2021) of one or few species.

The flight season of Southern species of *Somatochlora* begins in late August and continues into late September (Walker, 1925). Young adults will leave the vicinity after emergence and seek sheltered spots. Adults will typically fly at altitudes of 9-15m, although they will fly closer to the ground in search of prey or if wing musculature is not fully developed.

Imaginal life lasts approximately a month and a half. Adults will eat small insects, such as midges and black flies, while flying. Adults will seek a breeding place 2-3 weeks after emergence (Walker, 1925). Males will fly low, close to the surface of the water or bog where they alternate between rapid movements and hovering motionless. Females seldom fly low over water except when ovipositing. Mating rituals involve the males pouncing on females, both falling to the water, and separating. There are two types of ovipositors: rounded ovipositors directed towards the caudal end can be found in *S. arctica* and *S. alpestris* while spout-shaped ovipositors directed downwards can be found in *S. linearis, S. tenebrosa,* and *S. hineana*. Those with rounded ovipositors will strike the water with the end of their abdomen, releasing eggs into the water. Those with spout-shaped ovipositors will deposit their eggs near the water's edge while in flight (Walker, 1925).

Somatochlora nymphs' stadium duration depends on food and temperature conditions. Nymphs grow exceedingly slow with 6-7 molts per season and an overall nymphal development cycle of 4-5 years (Walker, 1925; Pintor and Soluk, 2006). Nymphs are ambush predators but have low hunting success and will seldom attack prey from a distance. Early-stage nymphs will feed on protozoa (*i.e., Euglena, Paramecium*) while later stages feed on larger aquatic invertebrates such as *Cyclops*, *Daphnia*, and oligochaetes. Nymphs can often be found in breeding places of adults in the benthic zone near the shore, where they will be frequently covered in mud or slime. *Somatochlora* nymphs prefer habitats with cool summer temperatures ranging from 16°C-20°C; consequently, nymphs prefer deep, lotic water systems (Walker, 1925). Before emergence, nymphs will climb onto wet moss above the water's edge. *Somatochlora* is a stenotopic genus; they are sensitive to anthropogenic disturbances and will often avoid dry streams and polluted waters.

Somatochlora dragonflies predominantly inhabit Palearctic and Nearctic realms, specifically subarctic/subalpine habitats (Walker, 1925). Most North American *Somatochlora* can be found in bog habitats from Lake Superior to Hudson Bay (Walker, 1925), but there are specific differences in habitat, such as *S. sahlbergi* preferring the tundra and *S. calverti* preferring sandy-forest streams (Dunkle, 2004; Schröter *et al.*, 2012). Of the 25 North American *Somatochlora* species, all have been designated a Species of Greatest Conservation Need in at least one U.S. state which may reflect the habitat degradation of fens and bogs characteristic of *Somatochlora* (Bried and Mazzacano, 2010).

The Hine's Emerald, *Somatochlora hineana* (Williamson), is a federally endangered dragonfly species (U.S. Fish and Wildlife Service, 2001) which is morphologically similar to *S. tenebrosa* (clamp-tipped emerald) (Williamson 1931). *Somatochlora hineana* requires three ecological requirements for appropriate habitat: 1) calcareous fens; 2) crayfish burrows for nymphal development; 3) shaded and unshaded pastures for foraging (Walker *et al.*, 2020). A fen is a type of wetland that takes millennia to develop and is difficult to restore from anthropogenic disturbance (Weixelman and Cooper, 2009). During drought periods, *S. hineana* nymphs will seek refuge in burrows created by the devil crayfish, *Cambarus diogenes,* which retain moisture when the heat dries the open channel (Pintor and Soluk, 2006). Nymphs are preyed upon mostly by *Aeshna* dragonflies (mosaic darners), dytiscids (predaceous diving beetles), and sialids (alderflies). *Somatochlora hineana* nymphs can and will be preyed upon by *C. diogenes* but seeking shelter in crayfish burrows leads to greater survivorship rates than risk of desiccation in open channels (Pintor and Soluk, 2006). *Somatochlora hineana* nymphs may occupy crayfish burrows despite flowing water (Pintor and Soluk, 2006).

The main range of *S. hineana* is from Southern Ontario to Wisconsin, Michigan, and Illinois (Craves *et al.*, 2022). A marginal population resides in the Ozarks in Missouri (Walker *et al.*, 2020). This marginal population has greater genetic diversity (that is, comprising an even distribution of older and younger mitochondrial haplotypes) than the core population of *S. hineana* residing in the Great Lakes (Walker *et al.*, 2020). Conservation of the marginal population may be critical for the preservation of ancestral haplotypes which may be beneficial for future adaptability (Walker *et al.*, 2020).

Geographically isolated populations are often genetically different (Avise, 2004). Population genetic architecture is influenced by a variety of forces including gene flow, random genetic drift, natural selection, mutational divergence, and genetic recombination. Gene flow via migration is important in vagile organisms with high mobility, such as insects. This is because genetic material can readily be exchanged between populations by movement of individuals or gametes. Gene flow may be affected by climate change, where species adapt to changing environmental conditions by changes in population range, thereby altering the likelihood of closely related species encountering (Arce-Valdés and Sánchez-Guillén, 2022). Interspecific hybridization may occur as a result of incomplete reproductive isolating barriers after secondary contact of closely related species (Arce-Valdés and Sánchez-Guillén, 2022). Genetic structure

assessments can elucidate the extent of introgression to a higher degree than morphological assessments alone (Avise, 2004), such as determining patterns of unidirectional hybridization (Solano *et al.*, 2018).

The aims of this study were: 1) to reconstruct multi-gene phylogenies using mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) for North American *Somatochlora* species; and 2) to investigate mitochondrial introgression indicative of heterospecific mating among North American *Somatochlora* by evaluating incongruence between phylogenies informed by mtDNA and nDNA. With the increasing number of studies indicating heterospecific mating is a common phenomenon in Odonata (Solano *et al.*, 2018; Kornová *et al.*, 2024), and that introgression runs deep in Odonata evolutionary history (Suvorov *et al.*, 2022), we seek evidence of *Somatochlora* interspecific hybridization or introgression. In addition, we use a novel molecular dataset which helps reevaluate the classification and relationships of North American *Somatochlora*. This study can provide a framework for future conservation studies, something especially pertinent to *Somatochlora* where all 25 North American species have been considered of great conservation need in at least one U.S. state (Bried and Mazzacano, 2010).

CHAPTER 2:
HETEROSPECIFIC MATING AMONG *SOMATOCHLORA*

Introduction

Mating between related species is a common occurrence among insects (Andersen *et al.*, 2019; San Jose *et al.*, 2023). Heterospecific mating attempts and heterospecific matings have been documented in the laboratory and field setting for the major insect orders, namely Hymenoptera, Diptera, Lepidoptera, Coleoptera, Odonata, and Orthoptera (Gröning and Hochkirch, 2008). Heterospecific matings are more likely to occur when heterospecifics are abundant and conspecifics are rare – a phenomenon known as the Hubbs Principle (Hubbs, 1955). Incomplete reproductive isolating barriers during allopatry occur frequently since time between speciation events is often much shorter than the window for hybridization (Chan and Levin, 2005). The effects of introgression will vary according to reproductive isolating barriers (*e.g.*, prezygotic vs. postzygotic) and modes of inheritance (*e.g.*, maternal vs paternal) For example, maternally inherited mitochondrial DNA will introgress more rapidly through ineffective prezygotic reproductive isolating barriers than paternal or biparental modes of inheritance (Chan and Levin, 2005). Prezygotic reproductive isolating barriers are especially sensitive to the proportion of immigrants; as a result, even a low migration rate can lead to high levels of introgression (Chan and Levin, 2005).

Odonata reproductive isolating barriers are mainly characterized by morphological and ethological barriers (Tennessen, 1982; Barnard *et al.*, 2017). Odonates have highly developed compound eyes; thus, they rely on visual stimuli such as sexual dimorphism in the form of color patterns and UV reflectance for mate recognition (Futahashi *et al.*, 2019). Other behaviors rely on tactile stimuli of genitalia (Tennessen, 1982). Males have elaborate claspers which are species specific (Figure 1) and thus are important for the identification of con- and heterospecifics when in copula (McPeek *et al.*, 2008). Male odonates use their terminal appendages to clasp the female's head. The females recognize conspecific males according to cerci morphology (Tennessen, 1982), but exceptions have been observed (Bick and Bick, 1981; Solano *et al.*, 2018). Indeed, genetic introgression runs deep in the evolutionary history of Odonata (Suvorov *et al.*, 2022). Heterospecific pairings have been recorded across families, mixed genera, and mixed species, including *Somatochlora* (Bick and Bick, 1981). However, evidence of heterospecific mating among *Somatochlora* is restricted to observational records and

morphological evidence of hybridization (Walker, 1925). For example, Walker noted a *Somatochlora* female of intermediate morphology; she had *S. cingulata* coloration and stature but with *S. albicincta* terminal appendage characteristics. In another instance, Bick and Bick (1981) observed tandem formation between *S. albicincta* (♂) and *S. hudsonica* (♀), but there was no direct evidence of hybridization. *Somatochlora sahlbergi* is known to hybridize with *S. hudsonica* and *S. albicincta* in northern Yukon where their ranges overlap (Cannings and Cannings, 1985). Potential *S. hineana* hybridization is restricted to accounts of *Somatochlora* specimens from the Missouri Ozarks whose identity was difficult to confirm (Monroe and Britten, 2014).

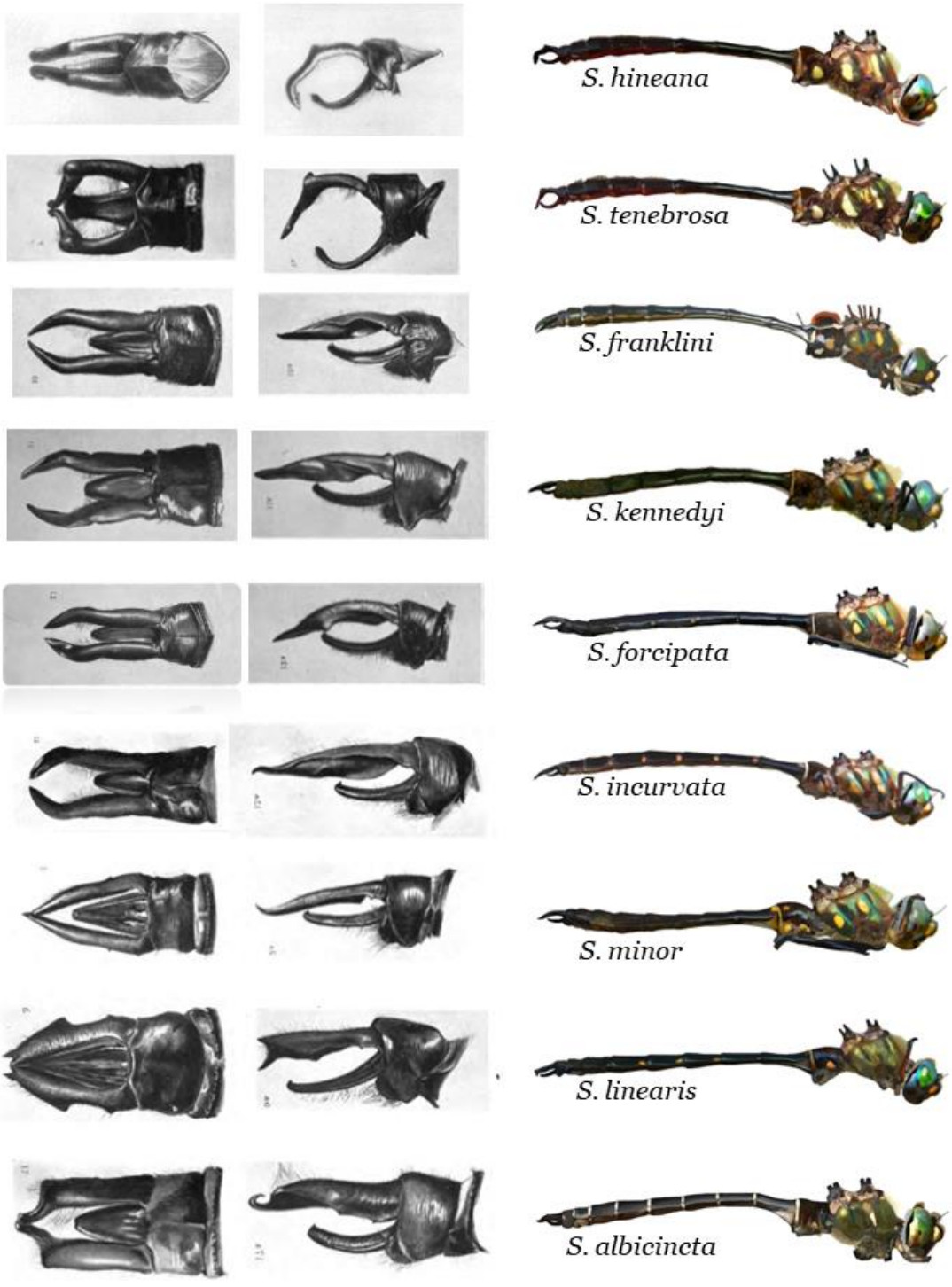

Figure 1 Illustrations of representative *Somatochlora* males used in this study and morphology of their cerci. The dorsal (left column) and lateral (center left column) view of the cerci are taken from Walker (1925) for all species except for *S. hineana* (Williamson, 1931). Illustrations were obtained from Mills (2015) with permission.

The use of mitochondrial genes for the discrimination of conspecifics and population genetic structure is well documented (Avise, 2004; Kohli *et al*., 2018). Despite its utility, several inherent properties of mtDNA limit conclusions drawn from the sole use of it. Mitochondrial genes can have more heterogeneity of site substitution variation than nuclear genes, leading to more homoplasy (Rubinoff and Holland, 2005). Nuclear genes have several qualities that are detrimental to their use in phylogenetics, such as heterozygosity, low substitution rates, low copy number, and paralogous loci. However, nuclear genes have less biased base composition than mitochondrial genes. Combined analysis of mtDNA and nDNA provides the most statistically robust and congruent phylogenies (Zhang and Hewitt, 2003; Rubinoff and Holland, 2005; Cameron, 2014). Two nDNA loci, Elongation Factor 1-α (*EF1-α*) and Internal Transcribed Spacer 2 (*ITS2*), as well as two mtDNA loci, NADH Dehydrogenase 3 (*ND3*) and Cytochrome C Oxidase I (*COI*) have been used for the discrimination of odonate species and other animal taxa (Pilgrim and Von Dohlen, 2008; Yao *et al.*, 2010; Bergmann *et al.*, 2013).

MtDNA and nDNA have distinct modes of inheritance which can provide insight into the degree of reproductive isolation for introgression asymmetries influenced by mate choice mechanisms (Solano *et al.*, 2018). MtDNA lineages go extinct after a speciation event four times faster than nuclear genes thus providing relative timing of population isolation and speciation events (Avise, 2004). Comparison of mtDNA and nDNA lineages can reveal four possible scenarios concerning speciation (Figure 2): (1) Non-monophyly for mtDNA and nDNA lineages suggests incomplete speciation; (2) monophyly of mtDNA and nDNA lineages suggests complete speciation; (3) monophyly of mtDNA but non-monophyly of nDNA lineages suggest recent speciation; (4) non-monophyly of mtDNA lineages but monophyly of nDNA lineages suggest gene flow after speciation. Observation of scenario (4) would provide phylogenetic evidence for heterospecific mating among dragonflies.

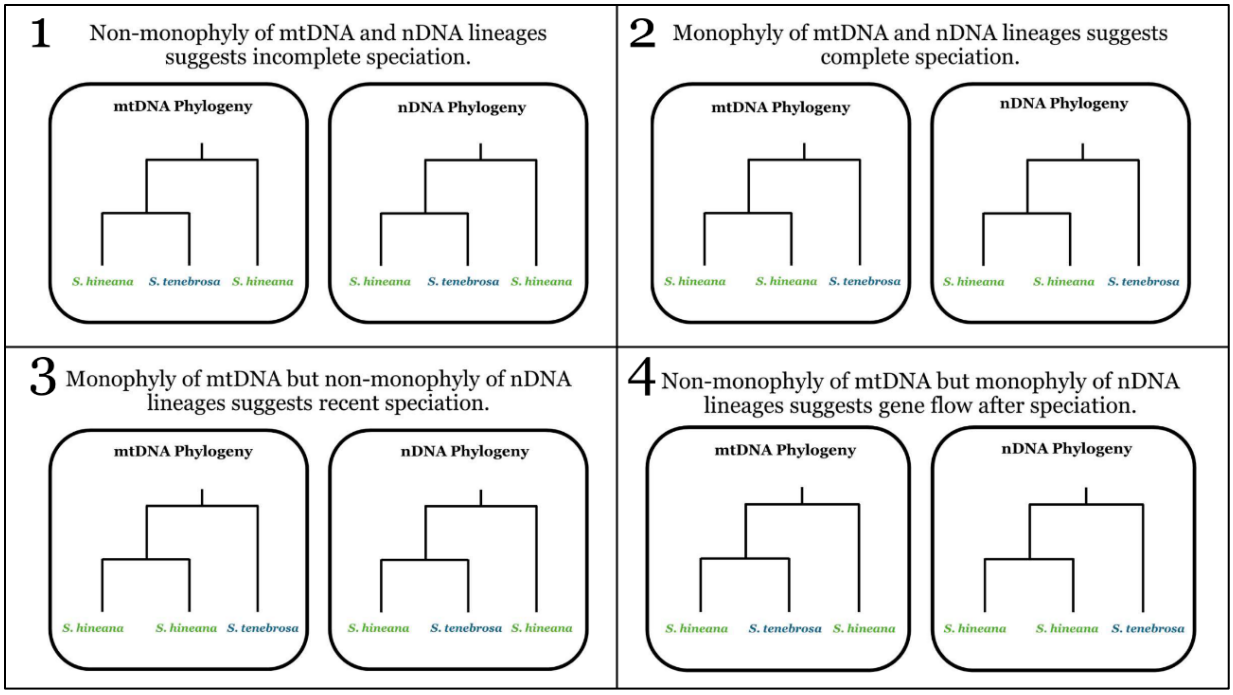

Figure 2 The four scenarios concerning speciation by comparison of mitochondrial- and nuclear based phylogenies.

The objective of this study is to investigate heterospecific mating between North American *Somatochlora* species as inferred from multi-gene phylogenies. *Somatochlora* are charismatic dragonflies with brown, green, blue, or black metallic bodies marked by yellow spots or stripes. These species, including *Somatochlora hineana* (the only federal-listed endangered dragonfly), are indicators of aquatic habitat quality because many inhabit ecologically sensitive wetlands such as fens (Vogt and Cashatt, 1994; Spoelstra and Post, 2023). Given the anecdotal observations of mating between *Somatochlora* species, we hypothesize potential gene transfer among species. Observation of non-monophyletic mtDNA lineages but monophyletic nDNA lineages between *Somatochlora* sister-species would indicate mtDNA introgression and suggest heterospecific matings. In addition, because this is the first phylogenetic study of North American *Somatochlora*, we broadly discuss the species relationships in reference to previous morphologically based taxonomy.

Materials and Methods

Specimens were obtained from museum collections and from the field. Most museum specimens were obtained from private and institutional collections. Field-collected specimens were enveloped, placed in acetone for 12-18 hours, and stored in a closed plastic container on silica gel. Vouchers were deposited in the A.J. Cook Arthropod Research Collection, MSU. In total, 108 specimens representing 31 *Somatochlora* species with 24/25 North American species and the remainder from Eurasia were included in this study (Table 1). Five taxa of Corduliidae (*Neurocordulia yasmakanensis*, *Dorocordulia libera*, *Cordulia shurtlefii*, *Epitheca spinigera*, and *Epitheca prínceps*) were selected as the outgroups.

For DNA extraction, tissue from a meso- or meta- leg was processed with the Qiagen DNeasy Blood and Tissue Kit following manufacturer protocols. Purified DNA from each specimen was used to amplify four target genes – Cytochrome C Oxidase 1 (*COI*), NADH Dehydrogenase 3 (*ND3*), Elongation Factor 1-α (*EF1-α*), and Internal Transcribed Spacer 2 (*ITS2*). PCR primers were selected from previously published primers or designed for this study (Table 2). PCR cocktails contained a mixture of 17.25 µL ddH20, 2.5 µL 10X PCR buffer (Qiagen), 1.0 µL 25 mM $MgCl_2$ (Qiagen), 0.5 µL dNTP mix (Qiagen), 2 µL template DNA, 0.25 µL HotStar Taq DNA polymerase (Qiagen), equating to a total volume of 25 µL. PCR was performed with a PTC-2000 MJ Research Thermocycler (MJ Research, Watertown, MA). Nuclear genes (*EF1-α*, *ITS2*) were initially denatured for 15 min at 95°C, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Final extension was at 72°C for 5 min. Alternative primer pairs were used and with the same thermocycler settings as stated previously except for a shorter extension time for 30 s at 72°C for samples that did not yield sufficient PCR product for *EF1-α*. Mitochondrial genes (*COI*, *ND3*) were initially denatured for 15 min at 96°C, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 45 s. Final extension was at 72°C for 5 min. PCR products were visualized using agarose gel electrophoresis and ethidium bromide illuminated under UV light. Following PCR visualization, samples were cleaned using ExoSAP-IT according to manufacturer protocol (Applied Biosytems by Thermo Fisher Scientific, Vilmus, Lithuania). Cleaned PCR products were sequenced (both strands via Sanger) at the Michigan State University Genomics Core Facility (East Lansing, MI).

Sense and antisense sequences were assembled and edited using Sequencher software version 5.0-7082 (Gene Codes Corporation). Consensus sequences were trimmed of primer sites and examined for ambiguous base calls and blasted In GenBank to check for potential contamination. Contamination or pseudogenes were not discovered. The resulting assembled sequences (base pairs: *EF1-α* = 618, *ITS2* = 417, *COI* =487, *ND3* = 541) were deposited in NCBI GenBank (Table 1). Protein coding genes were manually aligned given that nucleotide insertions/deletions and introns were not observed. ITS2 sequences were length variable, thus they were aligned using the EMBL-EBI Multiple Sequence Comparison by Log-Expectation program (MUSCLE) using the default settings (Madeira *et al.*, 2022).

A NEXUS file was created with the aligned sequences and use to infer phylogenies with PAUP* version 4.0a169 (Swofford, 2002). All DNA (the total dataset), mtDNA and nDNA data sets were analyzed. MtDNA and nDNA data were missing for some specimens and these were excluded from the genome specific phylogenetic analyses. Maximum parsimonious analyses consisted of 200 random stepwise addition heuristic searches with a tree-bisection-reconnection (TBR) branch-swapping algorithm. Gaps were treated as missing data. Jackknife branch support values (JK) were determined with 50%-character deletion using a 500 simple stepwise addition heuristic searches with a TBR branch swapping algorithm. Partition Bremer support was analyzed for the resulting strict consensus tree reconstructed for the total data set. TreeRot v2 (Sorenson, 1999) was used to build constraint trees for each node. Using the resulting constraint file, PAUP* was used to search for the most parsimony tree using the same search conditions explained above except branch swapping occurred on 500 best trees for each stepwise addition replicate. The averaged tree length for multiple trees found in each constraint tree search was subtracted from the partitioned lengths found in the unconstrained parsimony analysis. A negative value represented conflicting phylogenetic signal while a positive value represented supporting phylogenetic signal. All nodes unresolved in the strict consensus tree resulting from the simultaneous analysis of all data were zero.

Table 1. Voucher information for specimens used. "N/A" = missing sequence data.

				GenBank Accession No.			
Voucher	Genus	Species	Locality	*COI*	*ND3*	*EF1-α*	*ITS2*
COR_SHU1	*Cordulia*	*shurtleffii*	USA: Michigan: Marquette Co.	PP749106	PP751515	PP757551	PP748920
DOR_LIB1	*Dorocordulia*	*libera*	USA: Michigan: Marquette Co.	PP749107	PP751516	PP757552	PP748921
EPI_PRI1	*Epitheca*	*princeps*	USA: Michigan: Manistee Co.	N/A	PP751517	PP757553	PP748922
EPI_SPI1	*Epitheca*	*spinigera*	USA: Michigan: Marquette Co.	PP749108	PP751518	PP757554	PP748923
NEU_YAM1	*Neurocordulia*	*yamaskanensis*	USA: Michigan: Marquette Co.	PP749109	PP751519	PP757555	PP748924
SOM32	*Somatochlora*	*albicincta*	Canada: British Columbia	PP749110	N/A	N/A	PP748925
SOM91	*Somatochlora*	*albicincta*	Canada: New Brunswick	N/A	PP751520	PP757556	PP748926
SOM75	*Somatochlora*	*alpestris*	Norway	N/A	PP751521	N/A	N/A
SOM76	*Somatochlora*	*arctica*	Norway	N/A	N/A	N/A	PP748927
SOM28	*Somatochlora*	*brevicincta*	USA: Minnesota: Koochiching Co.	PP749111	PP751522	PP757557	PP748928
SOM112	*Somatochlora*	*calverti*	USA: Florida: Nassau Co.	PP749112	PP751523	PP757558	PP748929
SOM113	*Somatochlora*	*calverti*	USA: Florida: Gadsden Co.	PP749113	PP751524	PP757559	PP748930
SOM114	*Somatochlora*	*calverti*	USA: Florida: Leon Co.	PP749114	PP751525	PP757560	PP748931
SOM116	*Somatochlora*	*calverti*	USA: Florida: Liberty Co.	PP749115	PP751526	PP757561	PP748932
SOM33	*Somatochlora*	*cingulata*	Canada: Saskatchewan: Jade Lake	PP749116	PP751527	PP757562	PP748933
SOM106	*Somatochlora*	*elongata*	USA: Michigan: Marquette Co.	PP749117	PP751528	PP757563	PP748934
SOM107	*Somatochlora*	*elongata*	USA: Michigan: Marquette Co.	PP749118	PP751529	PP757564	PP748935
SOM119	*Somatochlora*	*elongata*	USA: Michigan: Marquette Co.	PP749119	PP751530	PP757565	PP748936
SOM120	*Somatochlora*	*elongata*	USA: Michigan: Marquette Co.	PP749120	PP751531	PP757566	PP748937
SOM121	*Somatochlora*	*elongata*	USA: Michigan: Baraga Co.	PP749121	PP751532	PP757567	PP748938
SOM35	*Somatochlora*	*elongata*	USA: Wisconsin: Vilas Co.	PP749122	N/A	PP757568	N/A
SOM68	*Somatochlora*	*elongata*	USA: Michigan: Mackinac Co.	PP749123	PP751533	N/A	PP748939
SOM8	*Somatochlora*	*elongata*	USA: Michigan: Marquette Co.	PP749124	PP751534	PP757569	N/A
SOM92	*Somatochlora*	*elongata*	USA: New Hampshire: Grafton Co.	N/A	PP751535	PP757570	PP748940
SOM93	*Somatochlora*	*elongata*	USA: New York: Broome Co.	PP749125	PP751536	PP757571	PP748941
SOM94	*Somatochlora*	*elongata*	USA: Maine: Somerset Co.	PP749126	PP751537	PP757572	PP748942
SOM104	*Somatochlora*	*ensigera*	USA: Michigan: Menominee Co.	N/A	PP751538	N/A	PP748943

Table 1 (cont'd)

Voucher	Genus	Species	Locality	GenBank Accession No.			
				COI	*ND3*	*EF1-α*	*ITS2*
SOM105	*Somatochlora*	*ensigera*	USA: Michigan: Menominee Co.	N/A	PP751539	PP757573	PP748944
SOM26	*Somatochlora*	*ensigera*	USA: Minnesota: Red Lake Co.	PP749127	PP751540	PP757574	PP748945
SOM115	*Somatochlora*	*filosa*	USA: Texas: Hardin Co.	PP749128	PP751541	PP757575	PP748946
SOM117	*Somatochlora*	*filosa*	USA: Texas: Hardin Co.	PP749129	PP751542	PP757576	PP748947
SOM68B	*Somatochlora*	*filosa*	USA: Florida: Bay Co.	PP749130	PP751543	PP757577	PP748948
SOM77	*Somatochlora*	*flavomaculata*	Lithuania	N/A	N/A	PP757578	PP748949
SOM102	*Somatochlora*	*forcipata*	USA: West Virginia: Tucker Co.	PP749131	PP751544	PP757579	PP748950
SOM16	*Somatochlora*	*forcipata*	USA: Wisconsin: Forest Co.	PP749132	PP751545	PP757580	PP748951
SOM37	*Somatochlora*	*forcipata*	USA: Vermont: Essex Co.	PP749133	PP751546	N/A	PP748952
SOM27	*Somatochlora*	*franklini*	USA: Minnesota: Koochiching Co.	PP749134	PP751547	PP757581	PP748953
SOM41	*Somatochlora*	*franklini*	USA: New Hampshire: Coos Co.	N/A	PP751548	N/A	PP748954
SOM66	*Somatochlora*	*franklini*	USA: Michigan: Crawford Co.	PP749135	PP751549	PP757582	PP748955
SOM14	*Somatochlora*	*franklini*	USA: Michigan: Alger Co.	PP749136	PP751550	PP757583	PP748956
SOM78	*Somatochlora*	*graeseri*	Russia: Sakhalin	PP749137	PP751551	PP757584	PP748957
SOM163	*Somatochlora*	*hineana*	USA: Michigan: Mason Co.	PP749138	PP751552	PP757585	PP748958
SOM118	*Somatochlora*	*hineana*	USA: Michigan: Mackinac Co.	PP749139	PP751553	PP757586	PP748959
SOM1	*Somatochlora*	*hineana*	USA: Michigan: Oceana Co.	PP749140	PP751554	PP757587	PP748960
SOM2	*Somatochlora*	*hineana*	USA: Michigan: Oceana Co.	PP749141	PP751555	PP757588	PP748961
SOM54	*Somatochlora*	*hineana*	USA: Michigan: Mackinac Co.	PP749142	PP751556	PP757589	PP748962
SOM55	*Somatochlora*	*hineana*	USA: Michigan: Mackinac Co.	PP749143	PP751557	PP757590	PP748963
SOM96	*Somatochlora*	*hineana*	USA: Wisconsin: Door Co.	N/A	N/A	PP757591	PP748964
SOM42	*Somatochlora*	*hudsonica*	Canada: British Columbia	PP749144	PP751558	N/A	PP748965
SOM97	*Somatochlora*	*hudsonica*	USA: Colorado: Larimer Co.	PP749145	PP751559	PP757592	PP748966
SOM3	*Somatochlora*	*incurvata*	USA: Michigan: Chippewa Co.	PP749146	PP751560	PP757593	PP748967
SOM60	*Somatochlora*	*incurvata*	USA: Michigan: Mackinac Co.	PP749147	PP751561	PP757594	PP748968
SOM70	*Somatochlora*	*incurvata*	USA: Michigan: Mackinac Co.	PP749148	PP751562	N/A	N/A
SOM95	*Somatochlora*	*incurvata*	Canada: Nova Scotia	PP749149	PP751563	PP757595	PP748969

Table 1 (cont'd)

Voucher	Genus	Species	Locality	GenBank Accession No.			
				COI	*ND3*	*EF1-α*	*ITS2*
SOM98	*Somatochlora*	*incurvata*	Canada: Nova Scotia	PP749150	PP751564	PP757596	PP748970
SOM100	*Somatochlora*	*kennedyi*	USA: Maine: Somerset Co.	PP749151	PP751565	PP757597	PP748971
SOM101	*Somatochlora*	*kennedyi*	USA: Alaska	PP749152	PP751566	PP757598	PP748972
SOM17	*Somatochlora*	*kennedyi*	USA: Wisconsin: Forest Co.	PP749153	PP751567	PP757599	PP748973
SOM43	*Somatochlora*	*kennedyi*	USA: Wisconsin: Eau Claire Co.	PP749154	PP751568	N/A	PP748974
SOM57	*Somatochlora*	*kennedyi*	USA: Michigan: Mackinac Co.	PP749155	PP751569	N/A	PP748975
SOM99	*Somatochlora*	*kennedyi*	USA: Maine: Lake Co.	PP749156	PP751570	PP757600	PP748976
SOM24	*Somatochlora*	*linearis*	USA: Texas: Jasper Co.	PP749157	PP751571	PP757601	N/A
SOM6	*Somatochlora*	*linearis*	USA: Michigan: Shiawassee Co.	PP749158	PP751572	PP757602	PP748977
SOM129	*Somatochlora*	*margarita*	USA: Texas: Marion Co.	PP749159	PP751573	PP757603	PP748978
SOM79	*Somatochlora*	*metallica*	Lithuania	PP749160	PP751574	PP757604	PP748979
SOM109	*Somatochlora*	*minor*	USA: Michigan: Alpena Co.	PP749161	PP751575	PP757605	PP748980
SOM18	*Somatochlora*	*minor*	USA: Wisconsin: Forest Co.	PP749162	PP751576	PP757606	N/A
SOM44	*Somatochlora*	*minor*	Canada: British Columbia	PP749163	PP751577	N/A	PP748981
SOM110	*Somatochlora*	*ozarkensis*	USA: Arkansas: Washington Co.	PP749164	PP751578	PP757607	PP748982
SOM71	*Somatochlora*	*ozarkensis*	USA: Oklahoma: McCurtain Co.	PP749165	PP751579	PP757608	N/A
SOM72	*Somatochlora*	*provocans*	USA: Mississippi: Stone Co.	N/A	PP751580	PP757609	N/A
SOM80	*Somatochlora*	*provocans*	USA: Florida: Washington Co.	PP749166	PP751581	PP757610	PP748983
SOM45	*Somatochlora*	*semicircularis*	Canada: British Columbia	PP749167	PP751582	N/A	PP748984
SOM46	*Somatochlora*	*semicircularis*	Canada: British Columbia	PP749168	PP751583	PP757611	N/A
SOM81	*Somatochlora*	*semicircularis*	USA: Idaho: Idaho Co.	PP749169	PP751584	PP757612	PP748985
SOM82	*Somatochlora*	*septentrionalis*	Canada: British Columbia	N/A	N/A	N/A	PP748986
SOM10	*Somatochlora*	*tenebrosa*	USA: Michigan: Hillsdale Co.	PP749170	PP751585	PP757613	PP748987
SOM11	*Somatochlora*	*tenebrosa*	USA: Michigan: Benzie Co.	PP749171	PP751586	PP757614	PP748988
SOM111	*Somatochlora*	*tenebrosa*	USA: Indiana: Johnson Co.	PP749172	PP751587	PP757615	PP748989
SOM12	*Somatochlora*	*tenebrosa*	USA: Michigan: Benzie Co.	PP749173	PP751588	N/A	PP748990
SOM164	*Somatochlora*	*tenebrosa*	USA: Michigan: Mason Co.	PP749174	PP751589	N/A	PP748991

Table 1 (cont'd)

Voucher	Genus	Species	Locality	GenBank Accession No.			
				COI	*ND3*	*EF1-α*	*ITS2*
SOM165	*Somatochlora*	*tenebrosa*	USA: Michigan: Mason Co.	N/A	N/A	N/A	PP748992
SOM19	*Somatochlora*	*tenebrosa*	USA: New York: Broome Co.	PP749175	PP751590	PP757616	PP748993
SOM21	*Somatochlora*	*tenebrosa*	USA: Oklahoma: McCurtain Co.	PP749176	PP751591	PP757617	N/A
SOM22	*Somatochlora*	*tenebrosa*	USA: Oklahoma: McCurtain Co.	PP749177	PP751592	PP757618	PP748994
SOM30	*Somatochlora*	*tenebrosa*	USA: Vermont: Washington Co.	PP749178	PP751593	PP757619	PP748995
SOM31	*Somatochlora*	*tenebrosa*	USA: Wisconsin: Sauk Co.	PP749179	PP751594	PP757620	PP748996
SOM56	*Somatochlora*	*tenebrosa*	USA: Michigan: Lenawee Co.	PP749180	PP751595	N/A	PP748997
SOM83	*Somatochlora*	*tenebrosa*	USA: Pennsylvania: Huntingdon Co.	PP749181	PP751596	PP757621	PP748998
SOM84	*Somatochlora*	*tenebrosa*	USA: Tennessee: Franklin Co.	PP749182	PP751597	PP757622	PP748999
SOM85	*Somatochlora*	*tenebrosa*	USA: Kentucky: Carter Co.	PP749183	PP751598	PP757623	PP749000
SOM86	*Somatochlora*	*tenebrosa*	Canada: Nova Scotia	PP749184	PP751599	PP757624	PP749001
SOM9	*Somatochlora*	*tenebrosa*	USA: Michigan: Manistee Co.	PP749185	PP751600	PP757625	PP749002
SOM87	*Somatochlora*	*uchidai*	Japan	PP749186	PP751601	PP757626	PP749003
SOM88	*Somatochlora*	*viridiaenea*	Japan	N/A	N/A	N/A	PP749004
SOM49	*Somatochlora*	*walshii*	USA: Maine: Washington Co.	PP749187	PP751602	N/A	PP749005
SOM69	*Somatochlora*	*walshii*	USA: Michigan: Mackinac Co.	PP749188	PP751603	N/A	N/A
SOM7	*Somatochlora*	*walshii*	USA: Michigan: Marquette Co.	PP749189	PP751604	PP757627	PP749006
SOM89	*Somatochlora*	*walshii*	Canada: Nova Scotia	PP749190	PP751605	PP757628	PP749007
SOM90	*Somatochlora*	*whitehousei*	Canada: British Columbia	PP749191	N/A	PP757629	PP749008
SOM122	*Somatochlora*	*williamsoni*	USA: Michigan: Baraga Co.	PP749192	PP751606	PP757630	PP749009
SOM123	*Somatochlora*	*williamsoni*	USA: Michigan: Marquette Co.	PP749193	PP751607	PP757631	PP749010
SOM52	*Somatochlora*	*williamsoni*	Canada: Saskatchewan: Jade Lake	PP749194	PP751608	PP757632	PP749011
SOM59	*Somatochlora*	*williamsoni*	USA: Michigan: Mackinac Co.	PP749195	PP751609	PP757633	N/A
SOM64	*Somatochlora*	*williamsoni*	USA: Michigan: Marquette Co.	PP749196	PP751610	PP757634	PP749012
SOM67	*Somatochlora*	*williamsoni*	USA: Michigan: Mackinac Co.	PP749197	PP751611	N/A	N/A
SOM73	*Somatochlora*	*williamsoni*	USA: Michigan: Mackinac Co.	PP749198	PP751612	PP757635	N/A

Table 1 (cont'd)

				GenBank Accession No.			
Voucher	Genus	Species	Locality	*COI*	*ND3*	*EF1-α*	*ITS2*
SOM74	*Somatochlora*	*williamsoni*	USA: Michigan: Shiawassee Co.	PP749199	PP751613	N/A	N/A

Table 2. Primer sequences.

Gene	Primers	Sequence	Annealing Temp.	Reference
COI	COI - 1709F	5' TAATTGGAGGATTTGGAAATTG 3'	45°	Kjer *et al.* (2001)
	COI - 2191R	5' CCYGGTARAATTARAATRTARACTTC 3'	45°	Kjer *et al.* (2001)
ND3	TG-J-5584	5' AGTATATTTGACTTCCAATC 3'	45°	Beckenbach *et al.* (2008)
	TN-N-6160	5' TCAATTATATCATTAACAGTGA 3'	45°	Beckenbach *et al.* (2008)
ITS2	CAS5p8sFc_Odon	5' TGAACATCGACATTTYGAACGCACAT 3'	55°	Ji *et al.* (2003)
	CAS28sB1d_Odon	5' TTCTTTTCCTCCSCTTAYTRATATGCTTAA 3'	55°	Ji *et al.* (2003)
EF1-α	EF1Rf_Odonate	5' GGAGAATTCGAAGCTGGTATCTC 3'	55°	Pilgrim and Von Dohlen (2008)
	EF1Ra_Odonate	5' GACACGTTCTTCACGTTGAAACC 3'	55°	Pilgrim and Von Dohlen (2008)
	Som EF1a A FW	5' CACTCCTCGCTTTCACTCTT 3'	55°	Designed in this study
	Som EF1a A REV	5' GCACTTTCCGTCAGCATTTC 3'	55°	Designed in this study
	Som EF1a B FW	5' GATGGAAGGTGGAGCGTAAG 3'	55°	Designed in this study
	Som EF1a B REV	5' CTCTTGGAGAGCTTCGTGATG 3'	55°	Designed in this study

Results

Mitochondrial analysis included 1030 characters of which 282 (27.4%) were parsimony informative and recovered 100000 trees with a length of 969. The strict consensus tree was mostly resolved and recovered most species as monophyletic except for three instances (Figure 3). First, *S. hineana* was rendered non-monophyletic with respect to *S. tenebrosa*. *Somatochlora hineana* SOM1 was recovered in a supported clade (86 JK) with *S. tenebrosa* SOM11 and *S. tenebrosa* SOM12. This clade was sister to the rest of *S. hineana* with strong support (96 JK). The remaining *S. tenebrosa* specimens were recovered in a strongly supported (98 JK) clade sister to the S. *hineana* clade. Second, there was a lack of resolution within a clade comprised of *S. kennedyi, S. franklini, S. forcipata,* and *S. semicircularis*. These species were recovered in a polytomy. Within this polytomy occurred a strongly supported (93 JK) clade of *S. incurvata*. Third, *S. provocans, S. calverti,* and *S. filosa* were recovered as non-monophyletic. *Somatochlora filosa* was rendered paraphyletic with respect to *S. provocans* SOM80 and *S. calverti* SOM114. *Somatochlora provocans* SOM80 was recovered in a poorly supported clade (70 JK) with *S. filosa* and *S. calverti* SOM114. Jackknife support values were greater for internal branches and smaller for intraspecific clades.

Nuclear analysis considered 992 included characters of which 271 (27.3%) were parsimony informative and recovered 55000 trees with a length of 928. PCR and sequencing of the nDNA loci had a lower success as compared to the mtDNA loci, with failure for 22 specimens. Specimens missing either *EF1-α* or *ITS2* sequences were excluded from the analysis of nDNA loci. Most groups recovered as non-monophyletic in the mtDNA-informed phylogeny were recovered as monophyletic in the nDNA analysis (Figure 4). *Somatochlora hineana* was recovered as a monophyletic clade with poor support (66 JK). *Somatochlora kennedyi, S. incurvata, S. franklini,* and *S. forcipata* were each recovered as monophyletic with moderate to strong jackknife support values of 96, 84, 92, and 99, respectively. *Somatochlora tenebrosa* was recovered unresolved but separate from *S. hineana,* with no specimens resolving except for *S. tenebrosa* SOM9 which was sister to *S. elongata* SOM92 in an unsupported clade. *Somatochlora filosa* was recovered as monophyletic with strong support (87 JK). *Somatchlora calverti* was recovered as monophyletic in an unsupported clade (51 JK). *Somatochlora provocans* was recovered sister to *S. calverti*. *Somatochlora walshii* recovered as monophyletic in the mtDNA-informed phylogeny but non-monophyletic in the nDNA analysis.

Combined analysis of mitochondrial and nuclear loci yielded the greatest resolution, for a total of 2022 characters of which 559 (27.6%) were parsimony-informative. There were 79000 most parsimonious trees (length=1995). Partition Bremer support values showed *COI* and *ITS2* to have the most positive clade support, while *ND3* and *EF1-α* had less support (Table 3). In general, *COI* and *ITS2* supported conspecific relationships, while *ND3* and *EF1-α* supported heterospecific and more inclusive clade relationships. Most groups were recovered as monophyletic (Figure 5). *Somatochlora hineana* resolved as monophyletic (1 BS), and most of *S. tenebrosa* resolved in a clade (1 BS) sister to *S. hineana*. The southern clade of *S. calverti + S. provocans + S. filosa + S. margarita + S. ozarkensis* was recovered in a well-supported (10 BS) but unresolved clade, with *S. filosa* resolving as monophyletic (2 BS). *Somatochlora linearis* recovered in a clade (1 BS) sister to southern clade. *Somatochlora ensigera* recovered in a strongly supported (6 BS) clade outside of *S. linearis*. *Somatochlora minor, S. elongata, S. williamsoni,* and *S. walshii* were recovered as monophyletic with Bremer support values of 1, 1, 4, and 4, respectively. *Somatochlora graeseri* and *S. uchidai* were recovered as sister species with strong support (27 BS). *Somatochlora incurvata* and *S. forcipata* were recovered as sister each other (1 BS). *Somatochlora franklini* and *S. kennedyi* were recovered as sister to each other (4 BS). This clade of *S. incurvata, S. forcipata, S. franklini, S. kennedyi,* and *S. semicircularis* was recovered with strong support (17 BS). A clade containing *S. brevicincta, S. albicincta, S. hudsonica, S. cingulata, S. whitehousei, S. septentrionalis, S. alpestris,* and *S. arctica* was recovered with moderate support (3 BS). *Somatochlora albicincta* and *S. hudsonica* were recovered as monophyletic with strong support, with Bremer support values of 4 and 7, respectively.

There were a few instances of nonmonophyly (Figure 5). *Somatochlora hineana* rendered *S. tenebrosa* paraphyletic, with two *S. tenebrosa* specimens (SOM11 & SOM 12) grouping with *S. hineana* at node 9. Mitochondrial genes (*COI + ND3*) provided conflicting or little support for node 9 (Table 3). *ITS2* provided most support for this node. *Somatochlora kennedyi, S. franklini,* and *S. forcipata* each resolved as monophyletic. In the case of *S. franklini* and *S. forcipata, ITS2* lent the most support (Table 3). In the case of *S. kennedyi, COI* and *ND3* lent the most support. *Somatochlora filosa* resolved as monophyletic with *EF1-α* contributing most to this clade. *S. provocans, S. calverti, S. margarita,* and *S. ozarkensis* were recovered in a polytomy.

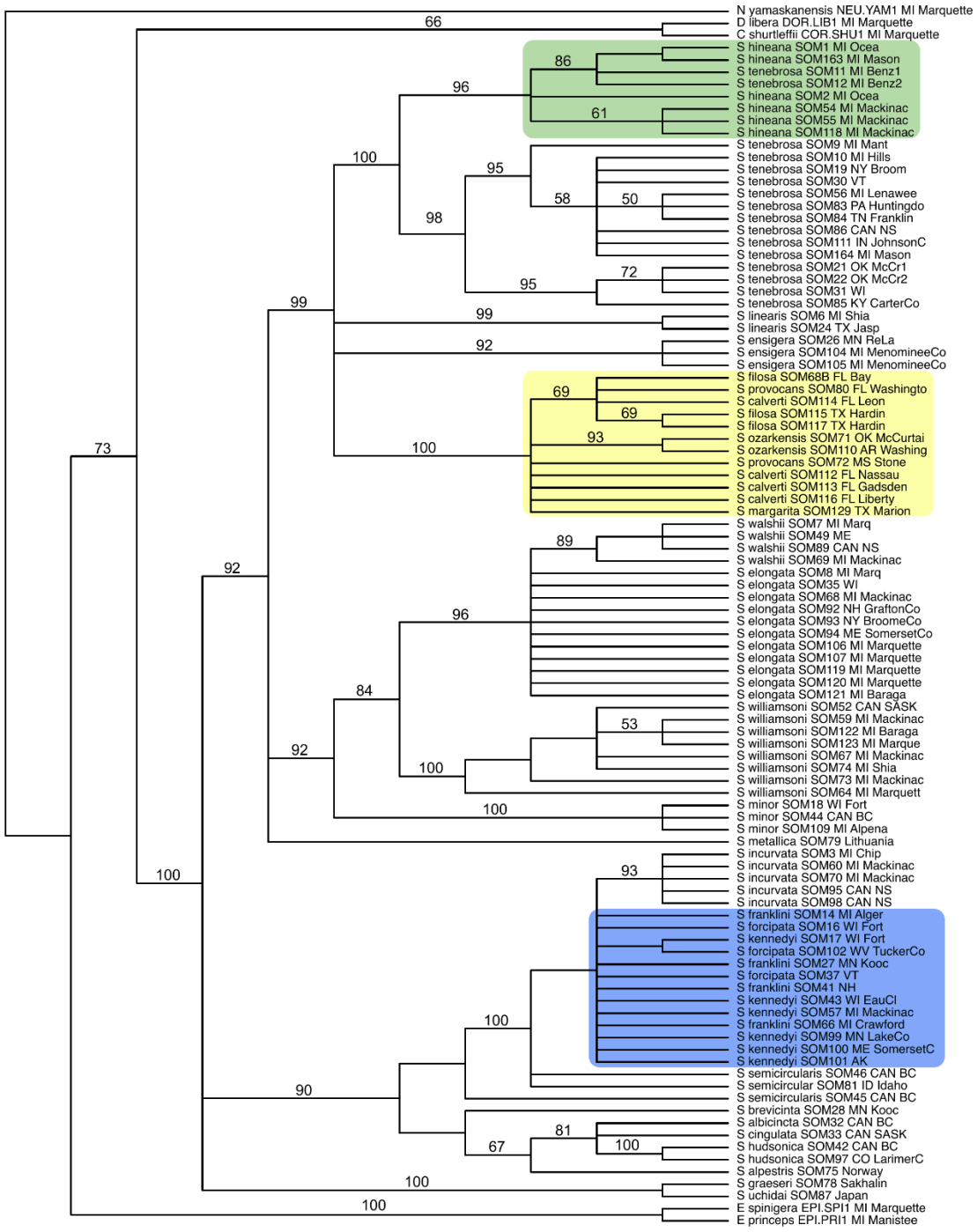

Figure 3 Rooted mitochondrial DNA (*COI* + *ND3*) strict consensus tree of 100000 most parsimonious trees for 100 *Somatochlora* specimens. Numbers above branches indicate jackknife support values greater than 50. Highlighted clades show mito-nuclear discordance and correspond to highlighted clades in Figure 4.

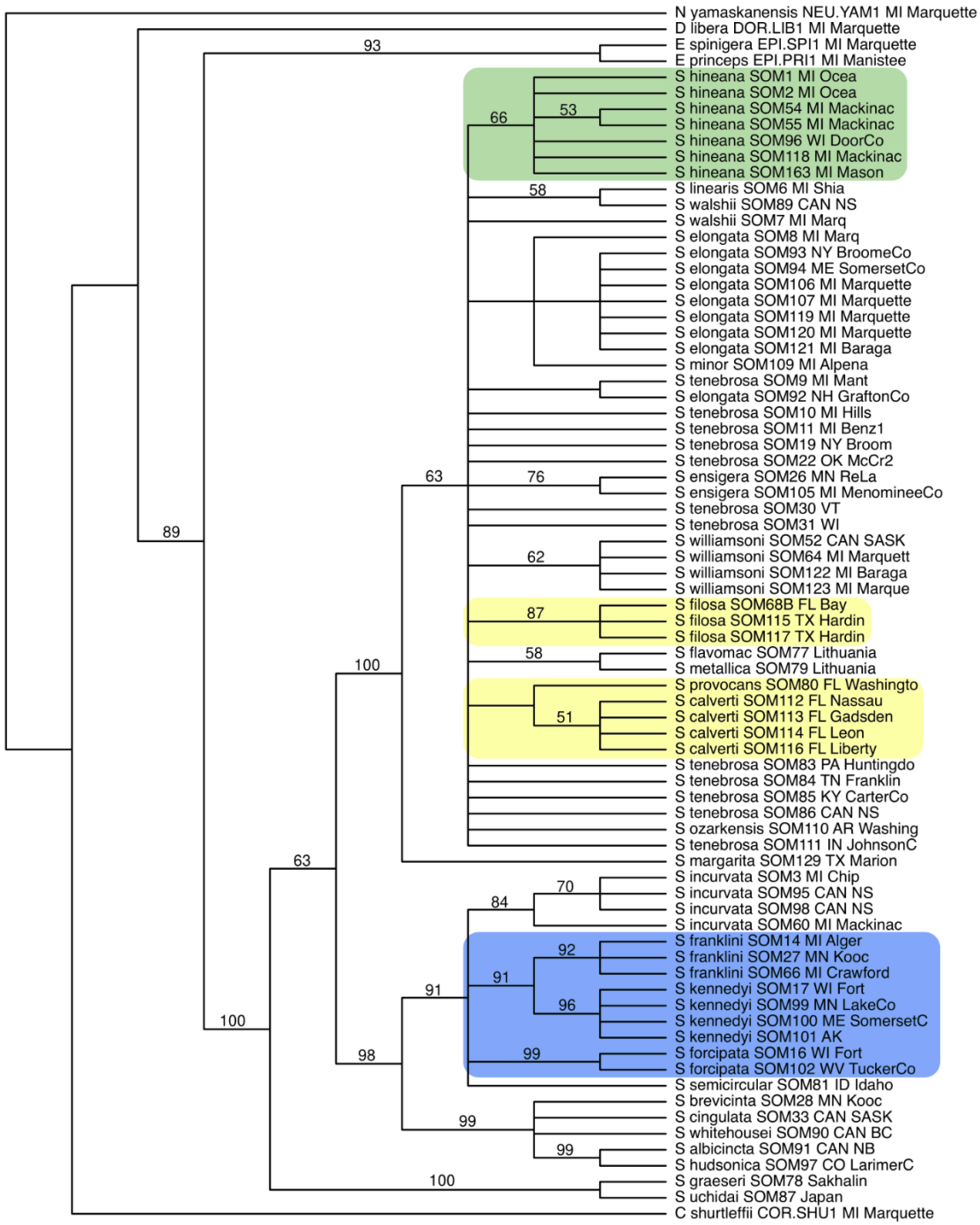

Figure 4 Rooted nuclear DNA (*EF1-α* + *ITS2*) strict consensus tree of 55000 most parsimonious trees for 86 *Somatochlora* specimens. Numbers above branches indicate jackknife support values greater than 50. Highlighted clades show mito-nuclear discordance and correspond to highlighted clades in Figure 3.

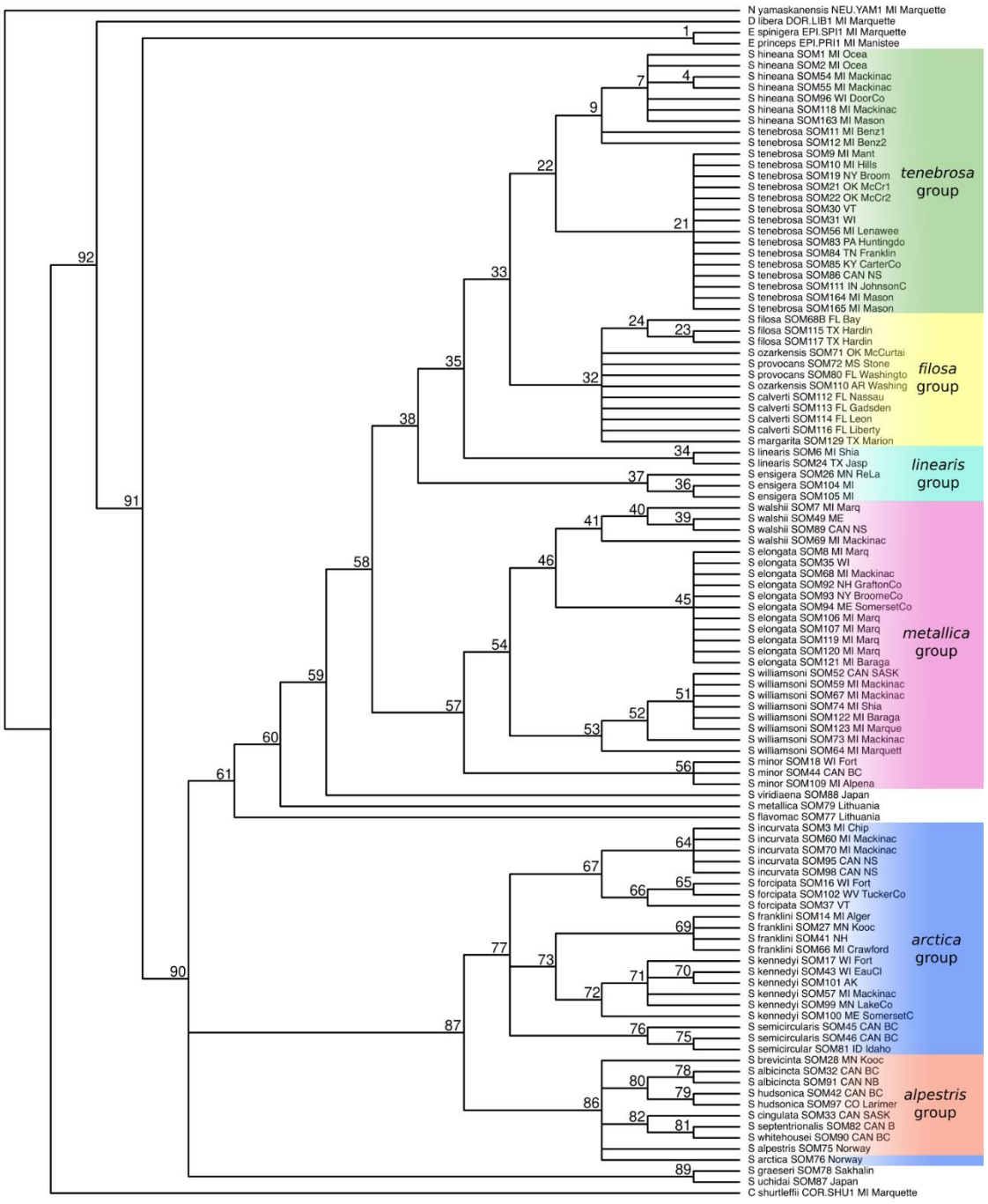

Figure 5 Rooted strict consensus tree of 79000 most parsimonious trees for 103 *Somatochlora* specimens using all data (*COI* + *ND3* + *EF1-α* + *ITS2*). Numbers indicate nodes corresponding to Table 3. Colored clades indicate prior taxonomic groups following Walker's (1925) group classification.

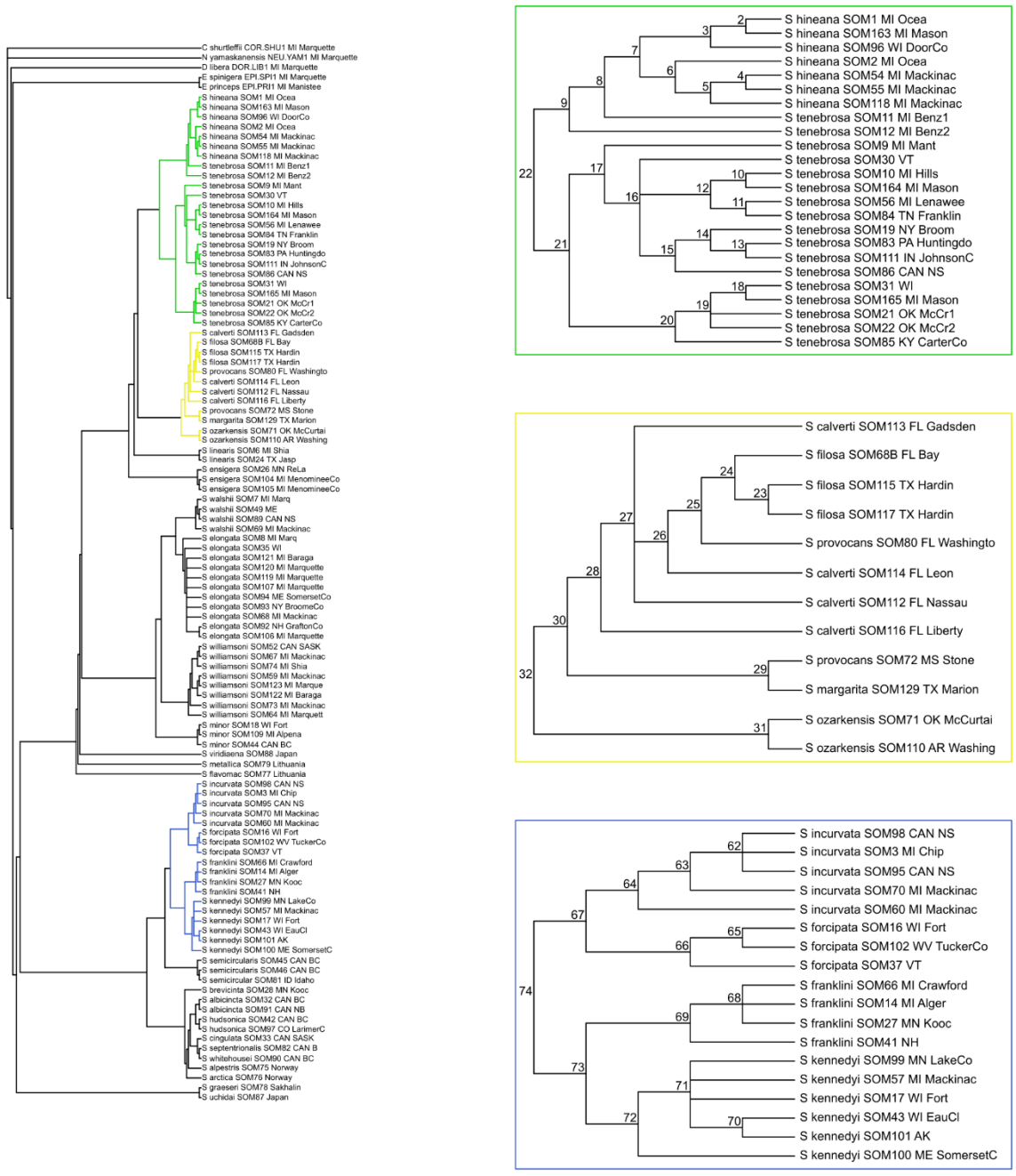

Figure 6 One of 79000 most parsimonious trees for 103 *Somatochlora* specimens using all data (*COI* + *ND3* + *EF1-α* + *ITS2*). Colored clades on the left correspond to the enlarged clades on the right. Numbers indicate nodes corresponding to Table 3.

Table 3. **Partition Bremer Support values.** Node numbers correspond to Figure 5 and Figure 6. Nodes highlighted in grey correspond to zero net support.

Node	*COI*	*ND3*	*EF1-α*	*ITS2*	Total BS
Node 1	-0.313636	23.6	4.909091	1.804545	30
Node 2	0.722727	0.163636	-0.690909	-0.195455	0
Node 3	0.512662	-0.036039	-0.033117	-0.443506	0
Node 4	1.259091	-0.557071	-0.087879	0.385859	1
Node 5	0.009091	0.254545	-0.218182	-0.045455	0
Node 6	0.575758	-0.191515	0.118788	-0.50303	0
Node 7	0.070202	-3.062626	1.30101	2.691414	1
Node 8	1.109091	-0.574848	0.085455	-0.619697	0
Node 9	-0.990909	0.281818	0.345455	1.363636	1
Node 10	2.109091	-0.051515	-1.454545	-0.60303	0
Node 11	0.699091	-0.204848	-0.081212	-0.41303	0
Node 12	0.982424	-0.301515	0.098788	-0.779697	0
Node 13	1.424091	-0.828182	-0.034545	-0.561364	0
Node 14	0.971591	-0.443182	-0.004545	-0.523864	0
Node 15	0.903535	-0.568182	0.20101	-0.536364	0
Node 16	-0.457576	0.515152	0.045455	-0.10303	0
Node 17	1.192424	-0.624848	0.198788	-0.766364	0
Node 18	0.880966	-0.468182	0.020455	-0.433239	0
Node 19	0.105245	-0.002797	0.160839	-0.263287	0
Node 20	0.594091	-0.533182	0.175455	-0.236364	0
Node 21	1.299091	-0.458182	-0.374545	0.533636	1
Node 22	1.484091	-0.643182	0.295455	-0.136364	1
Node 23	1.336364	1.1	0.127273	-0.563636	2
Node 24	0.259091	-0.312626	2.345455	-0.291919	2
Node 25	0.989091	-0.364848	-0.054545	-0.569697	0
Node 26	1.201948	0.31039	-1.225974	-0.286364	0
Node 27	0.304545	0.059091	-0.081818	-0.281818	0
Node 28	0.086014	-0.256643	0.345455	-0.174825	0
Node 29	0.444091	-0.428182	0.345455	-0.361364	0
Node 30	0.569091	-0.458182	0.345455	-0.456364	0
Node 31	0.697552	-0.418182	0.345455	-0.624825	0
Node 32	4.548377	3.521104	3.066883	-1.136364	10
Node 33	4.326738	-4.653476	2.313102	-0.986364	1
Node 34	0.909091	0.381818	0.345455	0.363636	2
Node 35	4.750758	-5.080682	2.549621	-1.219697	1
Node 36	3.694805	-3.43961	1.738312	-0.993506	1
Node 37	0.730519	2.538961	0.002597	2.727922	6
Node 38	-0.390909	1.081818	-1.054545	1.363636	1

Table 3 (cont'd)

Node	*COI*	*ND3*	*EF1-α*	*ITS2*	Total BS
Node 39	0.647552	0.627972	0.299301	-0.574825	1
Node 40	0.569091	0.595152	0.078788	-0.24303	1
Node 41	0.504545	0.131818	-0.263636	0.627273	1
Node 42	1.479091	0.191818	-0.874545	-0.796364	0
Node 43	0.112424	-0.058182	0.118788	-0.17303	0
Node 44	0.009091	0.049675	0.152597	-0.211364	0
Node 45	0.786869	0.248485	-0.276768	0.241414	1
Node 46	2.909091	-1.618182	0.345455	-0.636364	1
Node 47	0.142424	0.248485	-0.087879	-0.30303	0
Node 48	0.459091	0.063961	-0.175974	-0.347078	0
Node 49	0.349091	-0.338182	0.125455	-0.136364	0
Node 50	0.615758	-0.118182	0.212121	-0.709697	0
Node 51	1.390909	-0.190909	0	-0.2	1
Node 52	1.993706	-0.602797	0.176224	-0.567133	1
Node 53	2.959091	0.431818	0.245455	0.363636	4
Node 54	0.759091	1.231818	-1.354545	2.363636	3
Node 55	0.320629	-0.291259	0.153147	-0.182517	0
Node 56	1.286014	-0.591259	-0.185315	3.490559	4
Node 57	0.450758	-0.276515	0.295455	0.530303	1
Node 58	0.609091	-0.391259	0.306993	2.475175	3
Node 59	0.609091	-0.299432	0.195455	1.494886	2
Node 60	-0.903409	0.394318	0.320455	4.188636	4
Node 61	2.006459	-1.420813	2.950718	11.463636	15
Node 62	0.092424	0.048485	0.185455	-0.326364	0
Node 63	0.674476	-0.033566	-0.085315	-0.555594	0
Node 64	1.199091	4.295152	-0.107879	-0.386364	5
Node 65	1.124716	-0.068182	0.220455	-0.276989	1
Node 66	0.813636	1.718182	0	3.468182	6
Node 67	0.245202	-0.104293	-0.032323	0.891414	1
Node 68	0.579679	0.014171	-0.131016	-0.462834	0
Node 69	3.040909	-1.213636	-0.463636	0.636364	2
Node 70	0.259091	1.27028	-0.354545	-0.174825	1
Node 71	1.509091	0.021818	-0.014545	-0.516364	1
Node 72	2.559091	1.206818	0.345455	-0.111364	4
Node 73	3.092424	-0.674848	-0.981212	2.563636	4
Node 74	2.584091	-1.609848	0.328788	-1.30303	0
Node 75	0.809091	0.192929	0.034343	-0.036364	1
Node 76	0.092424	0.35404	-0.132323	0.685859	1
Node 77	1.959091	-1.618182	0.345455	16.313636	17

Table 3 (cont'd)

Node	*COI*	*ND3*	*EF1-α*	*ITS2*	Total BS
Node 78	0.136364	0.568182	0.3	2.995455	4
Node 79	0.609091	8.081818	-1.054545	-0.636364	7
Node 80	1.25	1.618182	0.036364	-0.904545	2
Node 81	-1.540909	0.431818	1.245455	0.863636	1
Node 82	-0.390909	-0.172348	1.045455	0.517803	1
Node 83	0.289091	-0.238182	-0.014545	-0.036364	0
Node 84	0.630519	0.017532	-0.097403	-0.550649	0
Node 85	0.223377	0.124675	0.288312	-0.636364	0
Node 86	-0.640909	3.581818	-0.054545	0.113636	3
Node 87	1.696591	7.419318	0.270455	-0.386364	9
Node 88	2.023797	-1.397594	-0.095722	-0.530481	0
Node 89	13.956313	2.773485	3.739899	6.530303	27
Node 90	1.909091	-1.618182	0.345455	56.363636	57
Node 91	2.426948	-3.503896	0.199026	9.877922	9
Node 92	3.115758	-3.541515	0.192121	2.233636	2
Total PBS	105.993686	24.808786	24.690885	114.506654	

Discussion

This study provides the first molecular evidence of heterospecific mating among *Somatochlora*. Our results highlighted three instances that suggest gene flow after speciation: 1) *S. hineana* + *S. tenebrosa*; 2) *S. kennedyi* + *S. forcipata* + *S. franklini*; 3) *S. calverti* + *S. provocans* + *S. filosa*. Mitochondrial haplotypes go extinct four times faster than nuclear haplotypes, thus after a speciation event, we would expect monophyly of the mtDNA lineage if there is monophyly of the nDNA lineage (Avise, 2004). Instead, there is nonmonophyly of the mtDNA lineages which suggests mtDNA introgression due to heterospecific mating. Combined analysis of mtDNA and nDNA can resolve relationships that are not found in separate analyses (Rubinoff and Holland, 2005); however, each partition differs in its incongruence across clades within trees and in magnitude (Damgaard and Cognato, 2003). Previous studies have shown that multiple genes can interact to recover phylogenetic signal and resolve clades (Cognato *et al.*, 2023). For unresolved nodes (BS = 0), *COI* provided the most support (27) while *ITS2* (-17) and *ND3* (-9) provided the most negative support. The large *COI* PBS values for unresolved nodes indicates support for non-monophyletic species while the negative values from *ND3* and *ITS2* indicate support for species monophyly. These partition incongruences suggest phylogenetic evidence of mitochondrial introgression, as exemplified by the positive support for unresolved nodes by mtDNA (*COI*) and negative support for these nodes by nDNA (*ITS2*). *EF1-α* provided the least support in resolved and unresolved nodes compared to the other genes, implying that *EF1-α* provided a general lack of phylogenetic information.

In odonates, rapid divergence of mechanical and tactile incompatibilities lead to sexual isolation and ecological divergence (Barnard *et al.*, 2017). Similar cerci morphology may prevent the reliable recognition of con- and heterospecifics (Paulson, 1974; Barnard *et al.*, 2017). *Somatochlora hineana* and *S. tenebrosa* have similar cerci morphology (Williamson, 1931). Both species share the following cercal morphological traits: 1) distinctly arched shaped cerci, with a dorsal process at about midlength; 2) convergence of the cerci (obtuse for *S. hineana* and acute for *S. tenebrosa*) on the descent; 3) setae on the concave side of the cerci; 4) epiprocts approximately three-fourths the length of the cerci (Walker, 1925; Williamson, 1931). The central habitat of *S. hineana* is found in the Great Lakes Region with a marginal population in Missouri (Craves *et al.*, 2022). *Somatochlora tenebrosa* is more common in eastern forests but can be found as far west as Missouri (Walker, 1925). Both species are partial to shady pastures

(Walker, 1925; Walker *et al.*, 2020). The comparatively abundant *S. tenebrosa* versus the endangered *S. hineana*, coupled with a shared habitat range, promotes an ecological phenomenon known as the Hubbs Principle (Hubbs, 1955), where a rare species is more likely to mate with an abundant heterospecific. Our results indicate the mating of *S. hineana* females with *S. tenebrosa* males, and not the reciprocal pairing, given that *S. hineana* mitochondrial haplotypes were recovered from *S. tenebrosa* males. If the less abundant *S. hineana* female is confronted by more *S. tenebrosa* males than her conspecific males, the female may be more inclined to mate with heterospecific males. Dragonfly females ultimately decide whether to copulate, and her decision to mate with heterospecific males may be influenced by increased male harassment (Tennessen, 1982; Kornová *et al.*, 2024). It is unknown if this asymmetrical mitochondrial gene flow, as observed for other insects (e.g. Cognato *et al.*, 1999), is prevalent in all areas of *S. hineana* and *S. tenebrosa* sympatry.

The polytomy within the mtDNA phylogeny and the monophyly within the nDNA phylogeny suggest gene flow between *S. kennedyi*, *S. forcipata*, and *S. franklini*. In general, this group + *S. incurvata* have forcipate cerci (in dorsal view) which curve inward in the distal third or beyond, with acute tips (in lateral view) (Needham *et al.*, 2000). These species share some cercal morphological traits (Walker, 1918). *Somatochlora kennedyi* shares the following traits with *S. franklini*: 1) cerci about as long as abdominal segments 9 and 10 combined; 2) lateral carinae with a small external basal tooth, at a slight angular bend about the middle; 3) length of epiprocts slightly more than half of cerci length. *Somatochlora forcipata* has the same cerci length in proportion to *S. kennedyi* and *S. franklini*; however, *S. forcipata* has more arched cerci, and a ventral carina near the basal fourth of the cerci from which extends a large blunt ventral tooth. However, like *S. kennedyi*, *S. forcipata* has flattened apices that are bluntly pointed, with the apices of *S. kennedyi* turning inward at an acute angle and the apices of *S. forcipata* turning inward at an obtuse angle. *Somatochlora kennedyi* and *S. franklini* are distributed widely across Canada and northeastern United States, while *S. forcipata* and its sister species, *S. incurvata*, are mostly restricted to southeastern Canada and northeastern United States (www.gbif.org). The shared habitat ranges of *S. kennedyi*, *S. franklini*, *S. forcipata*, and *S. incurvata* thus lend themselves to chance encounters between this group of closely related species.

Somatochlora incurvata was recovered as monophyletic and separate from *S. kennedyi*, *S. forcipata*, and *S. franklini* in both mtDNA and nDNA phylogenies. Considering we obtained

several specimens for each species (except *S. forcipata*) in relatively close proximity (Upper Peninsula of Michigan), it was surprising that mitochondrial introgression was not observed for *S. incurvata* given the species similar morphologies. In lateral view, the cerci of *S. incurvata* are less arched than those of *S. forcipata*, resembling those of *S. kennedyi* (Walker, 1925). Although the cerci are similar, they may act as a potential pre-mating barrier and hamper heterospecific mating among *S. incurvata* and its heterospecifics despite shared habitat range. Hamuli morphology also may serve as a premating barrier. The hamuli are a pair of male copulatory organs found on the venter of the second abdominal segment which contact the terminal reproductive organs of the female and function in species recognition (Watson, 1966). This group has similar morphology of the hamuli, especially between *S. incurvata* and *S. forcipata* (Walker, 1925). In general, the hamuli bend at almost a right angle, tapering abruptly to a blunt point. Local breeding habitat may better explain the monophyly of *S. incurvata*. *Somatochlora kennedyi*, *S. forcipata*, and *S. franklini* breed in fens with slow-flowing, spring-fed streams (Mead, 2021). *Somatochlora incurvata* breeding habitat is characterized by open sedge meadows where females prefer ovipositing in ephemeral pools (Mead, 2021; NatureServe, 2024). Consequently, *S. incurvata* may be found at breeding sites with comparatively lower amounts of water compared to its congeners (NatureServe, 2024), resulting in a decreased likelihood of heterospecific mating by local spatial isolation.

The coastal group – *S. calverti*, *S. provocans*, and *S. filosa* – lacked resolution in the mtDNA phylogeny. In the nDNA phylogeny, *S. filosa* was the only clade of the coastal group with moderate support (87 JK). An unsupported (51 JK) clade of *S. calverti* was recovered sister to *S. provocans*. In the combined analysis, *S. filosa* was the only resolved clade (2 BS). The rest of the coastal group + *S. margarita* + *S. ozarkensis* were recovered in a polytomy. This lack of resolution for both mtDNA and nDNA phylogenies indicates a dearth phylogenetic signal for the resolution of these species relationships. Still, the greater resolution of the nDNA phylogeny (*i.e., S. filosa* resolving separate from *S. calverti* and *S. provocans*) versus the mtDNA phylogeny suggests potential mitochondrial introgression. The polytomic coastal group had specimens that were collected from the Florida panhandle, a sympatric range for the three species. This group has varied cerci morphology. *Somatochlora provocans* is the most dissimilar of the group, with divergent cerci that enlarge in the proximal half, converging and tapering in the distal half. *Somatochlora calverti* and *S. filosa* share the following cercal traits: 1) cerci rather close together

at base, curving gently inwards in proximal third and 2) subparallel along middle length, somewhat swollen (Walker, 1925; Williamson and Gloyd, 1933). The cerci of *S. filosa* differ from *S. calverti*, with *S. filosa* cerci obtusely curving upwards in profile (Walker, 1925), while *S. calverti* has a sharp lateral angulation at midlength (Williamson and Gloyd, 1933). This morphological dissimilarity suggests their cerci would serve as an effective reproductive isolating barrier, because greater species-specific cerci would allow for discrimination between con- and heterospecifics. However, *S. filosa* is the most abundant species of the three along the Florida coast (www.gbif.org), which may lend itself to increased harassment of heterospecific *S. calverti* and *S. provocans* females by *S. filosa* males.

Although this study provides the first phylogenetic evidence for mitochondrial introgression among *Somatochlora*, heterospecific mating frequency and gene flow intensity remain unknown. In addition, the fitness consequences of introgression are not well understood. Understanding these factors is important for the conservation of the endangered *S. hineana* and other rare, range-restricted striped emeralds. These species may experience frequent mitochondrial and potential nuclear introgression because of increased sexual pressure to mate with heterospecifics thereby diluting the composition of genotypes. For *S. hineana,* the extirpation of populations from Ohio and Indiana further exacerbates this problem by severing gene flow between the Great Lakes and central US populations (Walker *et al.*, 2020). *Somatochlora kennedyi, S. forcipata,* and *S. franklini* provide a compelling argument that cerci morphology is not an infallible prezygotic reproductive isolating barrier for this predominantly Canadian group. Quantifying the amount of gene flow between these species will provide insight into population genetic architecture and the long-term effects of introgression for *Somatochlora* dragonflies. There is a marked lack of ecological studies focusing on *Somatochlora* (except *S. hineana*), so although species appear sympatric, there may be small-scale habitat associations among stenotopic *Somatochlora* that are acting as isolating barriers (Sánchez-Guillén *et al.*, 2012). Future evaluation of genetic variation at the genomic level will provide detailed measures of gene flow among *Somatochlora* as with other dragonflies (Higashikawa *et al.*, 2023). Detailed genetic studies have indicated several cases of potential species collapse engendered by hybridization and introgression (Rhymer and Simberloff, 1996), such as with the candy darter (Gibson *et al.*, 2019) and common raven (Kearns *et al.*, 2018). Detailed genetic studies will deepen our understanding of the evolutionary and ecological factors that maintain cohesion of

Somatochlora species and provide data needed for effective decision-making for the conservation of the endangered *S. hineana* (Craves *et al.*, 2022).

This study reconstructs the most comprehensive phylogenies of North American *Somatochlora* to date. In the combined data set, most groups resolved as in Walker's (1925) taxonomic revision of the genus. In this revision, Walker categorized North American *Somatochlora* into various groups according to similar morphology and distribution. There were six groups (Figure 5): 1) *tenebrosa* group – *S. tenebrosa*; 2) *filosa* group – *S. provocans* and *S. filosa*; 3) *linearis* group – *S. linearis* and *S. ensigera*; 4) *metallica* group – *S. minor, S. elongata, S. williamsoni,* and *S. walshii*; 5) *arctica* group – *S. franklini, S. kennedyi, S. forcipata, S. incurvata,* and *S. semicircularis*; 6) *alpestris* group – *S. whitehousei, S. septentrionalis, S. sahlbergi, S. albicincta, S. hudsonica,* and *S. cingulata*. In addition to these species, several Palearctic and more recently described species were included for phylogenetic analysis. For instance, Williamson (1931) described *S. hineana* and noted its close relation to *S. tenebrosa*. Bird (1933) and Donnelly (1962) described *S. ozarkensis* and *S. margarita*, respectively, and both authors concluded that their respective species belonged to the *filosa* group. Our results further corroborate Walker's hypothesis of *S. incurvata* and *S. forcipata* as sister-species. The original description of *S. brevicincta* stated its close relatedness to *S. albicincta* (Robert, 1954). Contrary to Robert's description, *S. albicincta* did not recover sister to *S. brevicincta* and was instead recovered sister to *S. hudsonica*. Instead of resolving with the *arctica* group like Walker (1925) predicted, *S. arctica* resolved with the *alpestris* group. Future phylogenetic study including genomic-level data and sampling of the world *Somatochlora* fauna will provide a more complete understanding of species relationships.

CHAPTER 3:
CONCLUSION

Somatochlora is the largest genus in the family Corduliidae whose breadth of systematic knowledge is limited. This study provides a framework for future research focused on North American *Somatochlora* and is the first to employ a novel dataset to reconstruct multi-gene phylogenies of the genus. There is phylogenetic evidence for heterospecific mating in the form of mitochondrial introgression among closely related *Somatochlora* species. However, much work needs to be done to fully understand the broad ecological impacts of long-term introgression.

Firstly, future studies utilizing genomic datasets of *Somatochlora* can provide further insight into the extent and directionality of mitochondrial and potential nuclear introgression, as well as provide greater evidence of true heterogeneity across mitochondrial clines rather than phylogenetic artifacts associated with the use of a few molecular markers. Obtaining a greater number of specimens can lead to a more complete picture of the genetic architecture of *Somatochlora* in the form of measuring gene flow among the nonmonophyletic clades. The unfortunate absence of *S. georgiana* (coppery emerald) in this study leaves questions regarding its relationship with its congeners. The cerci of *S. georgiana* most closely resemble that of *S. filosa*, but the dull, brown, non-metallic coloration of *S. georgiana* may function as an adequate visual cue that prevents mating with co-occurring closely related species. Future studies can investigate if certain clades of Palearctic *Somatochlora* display evidence of heterospecific mating. Gene flow studies powered by genomic data can help determine what the long-term impacts of introgression may be, especially for clades of disparate population sizes (*e.g.*, *S. hineana* and *S. tenebrosa*). Hybridization and introgression can threaten a rare species when it hybridizes with a common conspecific, a phenomenon common among animals (Rhymer and Simberloff, 1996). Phylogeographic studies would be especially informative as they can elucidate the historical range habitat of *Somatochlora* during the last glacial period. These types of studies would provide context for current species boundaries concerning both geographical distribution and morphological barriers.

Our explanation of mito-nuclear discordance revolves around morphological differences in genitalia (*i.e.*, cerci, hamules, epiproct) between closely related species. Detailed

morphometric analyses of these genitalia can quantify the architectural differences which would be useful for comparative studies of *Somatochlora* cerci. We used gbif.org occurrence data as a proxy for habitat range of specific *Somatochlora*; however, there may be fine-scale habitat differences associated with each species that may act as barriers to gene flow. At a more reductionist level, more accurate population surveys would lead to a more fine-tuned understanding of the population dynamics of this stenotopic genus, especially with climate-change-induced range fluctuations (Arce-Valdés and Sánchez-Guillén, 2022). For example, *S. incurvata* was previously thought to be rare, but more recent surveys revealed it was relatively common in its preferred habitat (Paulson, 2017). Previous studies have shown the caveats of solely using adult occurrences when making inferences about Odonata spatial distribution patterns, as often the breeding niches are more restricted than the niches of adults (Patten *et al.*, 2015). Specific ethological differences may also play a role in sexual selection and could provide context for the recovered phylogenies. Indeed, *S. incurvata* males are noted to be aggressive in securing a mate, chasing off other striped emeralds (Mead, 2021).

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