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Combining DNA sequences and morphology in systematics: testing the validity of the dragonfly species *Cordulegaster bilineata*

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Morphological and molecular techniques are rarely combined when answering questions of taxonomic validity. In this study, we combine morphological techniques with DNA sequences to determine the validity of the dragonfly species *Cordulegaster bilineata*. The two dragonfly species *C. bilineata* and *C. diastatops* are very similar in size, body color, and morphological characters, and due to these similarities, the status of *C. bilineata* as a valid species is in question. In this study we compare morphological measurements of males and internal transcribed spacer 1 (ITS-1) sequences of rDNA between the two taxa. The hamule measurements (where copulation occurs) of males show little difference between the taxa in question, but the anal appendage measurements (where the male first contacts the female) show marked divergence between the two taxa. Cluster analysis with these anal appendage measurements correctly assigns almost all individuals measured into their respective taxon. PCR amplification products of ITS-1 display a ~50 bp size difference between *C. bilineata* ($n = 4$) and *C. diastatops* ($n = 5$) regardless of collection site. Sequence data for these amplifications show 51 bp missing in one locus in the ITS-1 of *C. bilineata* relative to *C. diastatops*. A lone population of *C. diastatops* from Wisconsin has three individuals with ITS-1 products that match the size of both *C. bilineata* and *C. diastatops*. One individual from this population appears to yield two ITS-1 amplification products that match both *C. bilineata* and *C. diastatops*. Although this population may be evidence for hybridization between the two taxa, such hybridization is not necessarily sufficient to disqualify the validity of a separate species designation for *C. bilineata*. Morphology and ITS-1 sequences depict a high degree of divergence that is consistent with species-level differences. *Heredity* (2002) **89,** 184–190. doi:10.1038/sj.hdy.6800112

Keywords: *Cordulegaster*; ITS; rDNA; Cordulegastridae; Odonata; morphology

Introduction

Invertebrates often present special challenges to taxonomic studies. Species-level complications such as cryptic species and species complexes have become more apparent. Groups such as mites (Navajas *et al*, 1997), ticks (Wesson *et al*, 1993), and parasitic nematodes (Hung *et al*, 1997; Hoste *et al*, 1998) often lack sufficient morphological characters to allow for simple taxonomic comparison and identification. Many insect groups also share this lack of useful anatomical characters to aid in identification, including Homoptera (Cenis *et al*, 1993; Garcia, *et al*, 1998), Diptera (Wilkerson *et al*, 1995; Morrow *et al*, 2000), and Hymenoptera (Silva *et al*, 1999; Taylor and Szalanski, 1999). Molecular methods are becoming more useful and important as the need for taxonomic identification increases for many insect species due to their economic (Cenis *et al*, 1993) and medical (Wilkerson *et al*, 1995) and potential conservation significance. The 'lock and key' hypothesis, a staple of insect taxonomic work, where the fit of genitalia is thought to be strong evidence for distinction between species (Sota and Kubota, 1998), has become less credible in insect taxonomy (Porter and Shapiro, 1990), further complicating matters.

Purely morphological research in invertebrate taxonomic research is becoming rare (Leong and Hafernik, 1992; Lamberti and Ciancio, 1993; Verdyck *et al*, 1998) as molecular techniques become more popular. Techniques such as allozymes (Orr *et al*, 1994), RAPD-PCR (Wilkerson *et al*, 1995), RFLPs (Taylor and Szalanski, 1999) and DNA sequencing (Morrow *et al*, 2000) have all allowed researchers to look beyond morphological similarities and search for genetic differences that may help to solve taxonomic questions. Unfortunately, tension between molecular and morphological researchers has made studies that combine morphology and molecular analysis uncommon (eg, Orr *et al*, 1994; Navajas *et al*, 1997; Brust *et al*, 1998; Manguin *et al*, 1999; Raahauge and Kristensen, 2000).

In this study, we combine morphological and DNA sequencing approaches to address a question of taxonomic validity between two species of dragonflies (Odonata: Anisoptera), *C. bilineata* and *C. diastatops*. Since the separate designation of *C. bilineata* relative to *C. diastatops* (Carle, 1983), the status of *C. bilineata* as a true species has been in doubt (Glotzhober, 1997). The two species are very similar with only slight differences in

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body color, thoracic stripe width, and some minor morphological characters. These slight distinctions could be the result of simple variation within characters, environmental effects, or geographic variation within *C. diastatops* (Glotzhober, 1997). In the description of *C. bilineata*, Carle (1983) also places *C. diastatops*, *C. bilineata*, and a third species, *C. sayi*, into a restored genus, *Zoraena*. Our work does not address the validity of resurrecting the genus *Zoraena*, as other researchers have already considered it to be a synonym of *Cordulegaster* (Paulson and Dunkle, 1999). This study focuses only on the validity *of C. bilineata* as a separate species from *C. diastatops*.

The unique method of mating in odonates (see Corbet, 1999 for a review) creates some interesting issues when searching for morphological distinction between species. In dragonflies, the male uses the anal appendages on the tip of his abdomen (10th segment) to grasp the female's thorax. Mating then takes place as the female brings her abdomen underneath the male to contact the hamule region near the base of his abdomen (2nd and 3rd segments) where sperm transfer then occurs. In male odonates, the anal appendages and/or the hamules can be species specific (Corbet, 1999). Any taxonomic work on odonate species must include a careful consideration of both of the morphological regions involved with mating.

The purpose of this study is to integrate morphological measurements and DNA sequences as tools of taxonomy. This study is the first to combine morphological characters with DNA sequence data in species-level dragonfly taxonomy and has several objectives. First, we compare morphological measurements of both the anal appendages and the hamule region to look for potential divergence between *C. bilineata* and *C. diastatops*. As both of these structures are involved with copulation, we would expect selection pressure, sexual selection (Eberhard, 1985) or even drift to create divergence between two valid species in one or both of these regions. Second, we compare sequences of the first internal transcribed spacer region (ITS-1) of nuclear rDNA for differences between the two taxa. Although much of the molecular work in taxonomy has been concerned with mitochondrial genes (Simon *et al*, 1994), the ITS regions are becoming more popular as species-level divergence is common within these regions of DNA (Kuperus and Chapco, 1994; Silva *et al*, 1999). The final objective of this work is to combine both the morphological and genetic data to uphold or refute the validity of *C. bilineata* as a legitimate species.

Methods

Morphological data collection

In order to look for potential morphological differences between the two taxa, we measured 11 different male characters used either in gripping the female or in copulation. Male dragonflies are more common than females in collections and often have more useful characters for species determination (Leong and Hafernick, 1992). Corresponding female characters may exist on their thorax, but the number of female specimens was so low that comparison was not possible (less than 10 specimens total between the two taxa). We measured 70 specimens of *C. bilineata* from a large geographic range (Figure 1) that included several paratype specimens from the original

Figure 1 Map of collection sites from the eastern United States. Closed circles show the spread of *Codulegaster bilineata* sites, and X's show the spread of *C. diastatops* sites. The large, black triangle marks the 'problematic' Wisconsin population.

description of *C. bilineata*. We also measured 40 specimens of *C. diastatops* from a similarly sized geographic range (Figure 1). Specimens were identified using the key in Carle (1983) that uses morphological and color characters.

These measurements included six from the hamule region of abdominal segments two and three (where copulation occurs) and five measurements of the anal appendages (used to grasp the female prior to copulation) of segment 10 (Figure 2). The characters chosen also had the advantage of not being obscured from view by other structures. All measurements were made with an ocular micrometer on a dissecting scope and were estimated to the nearest half division (36.8 microns per division). Several structures are paired in

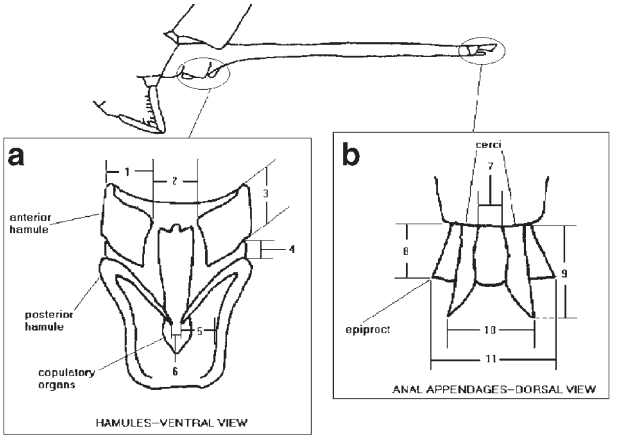

Figure 2 Morphological traits measured in *Cordulegaster bilineata* and *C. diastatops*. Both species are of similar body size. The hamule measurements (**a**) consisted of anterior hamule length (1) (AHL), anterior hamule to hamule distance (2) (AHHD), anterior hamule width (3) (AHW), anterior to posterior hamule distance (4) (APHD), posterior hamule distance from the genital fossil wall (5) (PHW), and the posterior hamule to hamule distance (6) (PHHD). The anal appendage measurements (**b**) consisted of the cercus to cercus proximal distance (7) (CPW), epiproct length (8) (EL), cerci length (9) (CL, cercus to cercus distal width (10) (CDW), and the epiproct width (11) (EDW).

individuals (cerci, anterior and posterior hamules), so right and left measurements were averaged to give a mean value for that individual. Morphological data were compared with *t*-tests and cluster analysis (UPGMA) using SAS software (v. 8.1).

Genetic data collection

High molecular weight genomic DNA was isolated from measured specimens and from several female specimens. For comparison against other members of the genus *Cordulegaster*, one individual each of *C. maculata* and *C. erronea* were also included. DNA was isolated from a leg of each individual using the QIAamp DNA Minikit following the Tissue Protocol (QIAGEN, Valencia, CA, USA). DNA samples were stored in the kit's buffer at 4°C until needed for polymerase chain reaction (PCR). Older museum specimens (before 1980) often failed to yield useful DNA for PCR amplification.

PCR took place in 20 μL volumes with reaction conditions of 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 (Promega Mg Free commercial buffer), 3 mM $MgCl_2$, 200 pM dNTPs, and 2 units of Promega Taq polymerase. Amplification was accomplished using primers 5′-TCAA CACGGGACCCAGGCCC-3′ (forward-18S) and 5′-CGATGATCAAGTGTCCTGCA-3′ (reverse-5.8S) to amplify the entire ITS-1 region. The 18S primer was developed comparing sequences of *Aeshna cyanea* from GenBank (accession no. X89481) with *Macromia taeniolata* (unpublished), while the 5.8S primer was developed solely from *M. taeniolata*. The reaction program was 1 cycle at 95°C for 3 min, 56°C for 20 sec, and 72°C for 35 sec followed by 34 cycles of 95°C for 30 sec, 56°C for 20 sec, and 72°C for 35 sec and a final extension of 72°C for 10 min. Amplification products were size fractionated on 1.5–2.0% agarose gels stained with ethidium bromide.

Sequencing reactions were performed with the ATBI Big Dye Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems. The reactions followed the protocols outlined in the accompanying manual using purified PCR product (~90 ng) and an internal primer (5′-GATTAGGTCCCTGCCCTTTG-3′). After ethanol precipitation, sequencing reactions were run through an ABI Prism 310 Genetic Analyzer. Generated sequences were aligned with CLUSTAL W 1.8 (Thompson *et al*, 1994). Nucleotide sequences for the ITS-1 of all taxa have been submitted to GenBank (accession nos. AY082597 to AY082601).

Results

Morphological measurements

Both the hamule and anal appendage measurements show high variation among and within the two taxa, as evident by the minimums, maximums, and standard deviations (Table 1). Satterthwaite *t*-tests (accounting for unequal variance) of all measurements show significant differences between the two taxa for AHW and PHW of the hamule measurements, and for all the anal appendage measurements (Table 1). As the data for some measurements did not fit a normal distribution, we used Wilcoxon Rank Sum test to check for significant differences between the two taxa. The same measurements from the *t*-test also show significant differences in the Wilcoxon Rank Sum test. Transforming the data did not give a normal distribution as several individuals often had identical values for any one measurement. The non-normal distribution of some measurements is most likely an artifact of the method of measurement (ocular micrometer), but the sample size of each measurement is large enough for the *t*-tests to be a valid comparison of the data sets.

We also used several different combinations of measurements in cluster analysis (UPGMA). No real differences were shown between *C. diastatops* and *C. bilineata* using only the hamule measurements. Individuals of both taxa are spread throughout the cluster dendrogram with no real pattern. When we used all the measurements in the analysis, the dendrogram has two clusters with *C. bilineata* individuals in one and a second cluster with *C. diastatops* individuals nested within a second cluster of *C. bilineata* individuals. Using only the anal appendage measurements, however, shows a large degree of morphological divergence between *C. bilineata* and *C. diastatops* (Figure 3). Each taxon forms its own cluster within the dendrogram with the exception of two stray individuals of *C. diastatops* that appear within the *C. bilineata* cluster. Using only the measurements EDW, CL, and CDW creates an identical dendrogram of divergence between the two groups.

In the original description of *C. bilineata*, Carle (1983) mentions that the ratio of EDW to CL is often below 1.3 for *C. bilineata* and greater than 1.3 for *C. diastatops*. Over 95% of the *C. bilineata* individuals of measured (67 of 70) have an EDW to CL ratio below 1.35 (mean: 1.19, mode: 1.23, median: 1.19). For *C. diastatops*, almost 95% of the individuals measured (36 of 38) have a ratio of 1.35 or above (mean: 1.57, mode: 1.63, median: 1.56).

ITS-1 sequences

PCR amplification and gel electrophoresis revealed a size difference of approximately 50 base pairs (bp) between *C. diastatops* and *C. bilineata* (Figure 4a). The only individuals with successful amplifications that did not conform to this size difference were three individuals from Spur Lake, WI (Figure 4b). These individuals were originally classified as *C. diastatops*, however, one individual had a band whose size corresponded to *C. bilineata*, one individual had a band that corresponded to *C. diastatops*, and one individual appears to have two bands that match both taxa. Otherwise, this size difference was apparent for 29 different individuals of *C. bilineata* and for 16 individuals of *C. diastatops*, all from a wide geographic range.

Sequence data for the 18S portion of the *Cordulegaster* species matched very closely with the *Aeshna cyanea* sequence (only four differences in over 500 bp of 18S sequence prior to the ITS-1). ITS sequences ranged in size from 271 to 336 bp for the four species of *Cordulegaster*, with 271 bp for *C. bilineata*, 286 bp for *C. maculata*, 323 to 327 bp for *C. diastatops*, and 336 bp for *C. erronea*. The four different individuals of *C. bilineata* (one from IL, two from different sites in OH, and one from MI) had identical ITS-1 sequences. Of the five individuals of *C. diastatops* sequenced, individuals from NY and MA had identical sequences, but one individual from MD has an extra GAGA relative to the other *C. diastatops* sequences (Figure 5).

Alignment of the ITS sequences (Figure 5) showed that the size difference between the PCR products of *C. bilineata* and *C. diastatops* was due to 51 bp missing from a

Table 1 Morphological character summary statistics for *Cordulegaster bilineata* and *C. diastatops*

Meas.	*C. bilineata*				*C. diastatops*				*Satterwaite t-test*	
	n	*Mean*	*SDEV*	*Coef. Var.*	*n*	*Mean*	*SDEV*	*Coef. Var.*	*df*	*P value*
AHL	70	878	44.0	0.050	40	890	38.5	0.043	103	0.1391
AHW	70	822	152.3	0.185	40	731	107.5	0.147	90.5	0.0004*
APHD	70	135	106.7	0.790	40	113	72.8	0.644	104	0.2001
PHW	70	649	115.3	0.178	38	587	119.4	0.203	73.8	0.0103
AHHD	70	764	141.2	0.185	40	718	136.4	0.190	83.7	0.0910
PHHD	70	388	73.4	0.189	38	389	98.4	0.253	59.8	0.9271
EDW	67	1819	95.7	0.053	40	2056	106.6	0.052	75.3	<0.0001*
EL	69	954	109.0	0.114	40	1022	112.7	0.110	79.4	0.0030*
CL	67	1535	90.8	0.059	36	1319	79.4	0.060	80.4	<0.0001*
CPW	68	753	84.7	0.112	39	897	118.4	0.132	60.6	<0.0001*
CDW	63	673	220.5	0.328	30	1103	203.6	0.185	61.8	<0.0001*

*Denotes significance at Bonferroni corrected $P = 0.0045$.

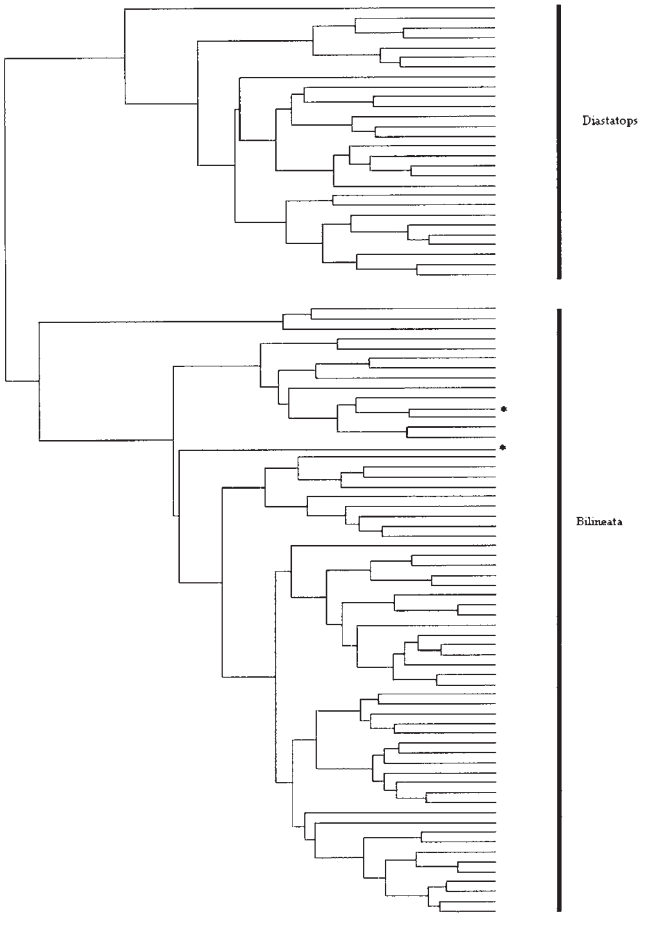

Figure 3 Cluster dendrogram of *Cordulegaster bilineata* and *C. diastatops* individuals using anal appendage measurements (EDW, EL, CL, CPW, and CDW). Two individuals of *C. diastatops* within the *C. bilineata* cluster are marked with asterisks.

single portion of the ITS-1 of *C. bilineata* relative to *C. diastatops*. Two T to G transversions and a C to T transition also existed between the two taxa. Comparison to the other two *Cordulegaster* species suggested that the missing 51 bp in *C. bilineata* most likely resulted from a deletion of those base pairs.

Discussion

Clearly, *C. bilineata* and *C. diastatops* have diverged morphologically. The contrast between morphology of the two cannot be explained by overall body size variation, as *C. bilineata* and *C. diastatops* are very similar in size. The two taxa may have similar hamule characteristics, but the anal appendage measurements of each species show large differences between the two. The high significance in several *t*-tests ($P < 0.0001$) in anal appendage measurements speaks to the degree of morphological divergence that exists between *C. bilineata* and *C. diastatops*. The divergence in anal appendages between morphologically similar species is not unusual, as these appendages are the first parts of the male that contact the female prior to mating (Corbet, 1999). Females odonates are known to reject males of other species based on this type of contact (Corbet, 1999). The EDW to CL ratio shows that almost all *C. bilineata* fall below the critical value of 1.35 and that most *C. diastatops* are above this value. When all the anal appendage measurements are taken as a group in cluster analysis, the degree of divergence between *C. bilineata* and *C. diastatops* becomes even more evident. The two taxa are also genetically distinct as shown by their ITS-1 sequences. The 51-bp deletion is striking evidence of genetic divergence between *C. bilineata* and *C. diastatops*. A transition and two transversions in the ITS-1 sequences are also significant evidence for genetic distance between *C. bilineata* and *C. diastatops*.

Although the morphological evidence alone may only be enough to relegate *C. bilineata* to subspecific status, the morphological differences coupled with the genetic data strongly suggest that *C. bilineata* be accepted as a valid species. Differences similar to that between *C. bilineata* and *C. diastatops* exist between other, well-accepted species of aphids and wasps (Fenton *et al*, 1998; Silva *et al*, 1999). ITS-1 sequence differences have been reported within a single species over a large geographic area (Vogler and DeSalle, 1994). The sequence differences between *C. bilineata* and *C. diastatops*, however, do not fit this pattern of within-species variation. The size difference in ITS-1 product remains constant for each taxon over a large geographic area (except for the three Wisconsin (WI) individuals). The individuals sequenced for *C. bilineata* come from a wide geographic range (Illinois to

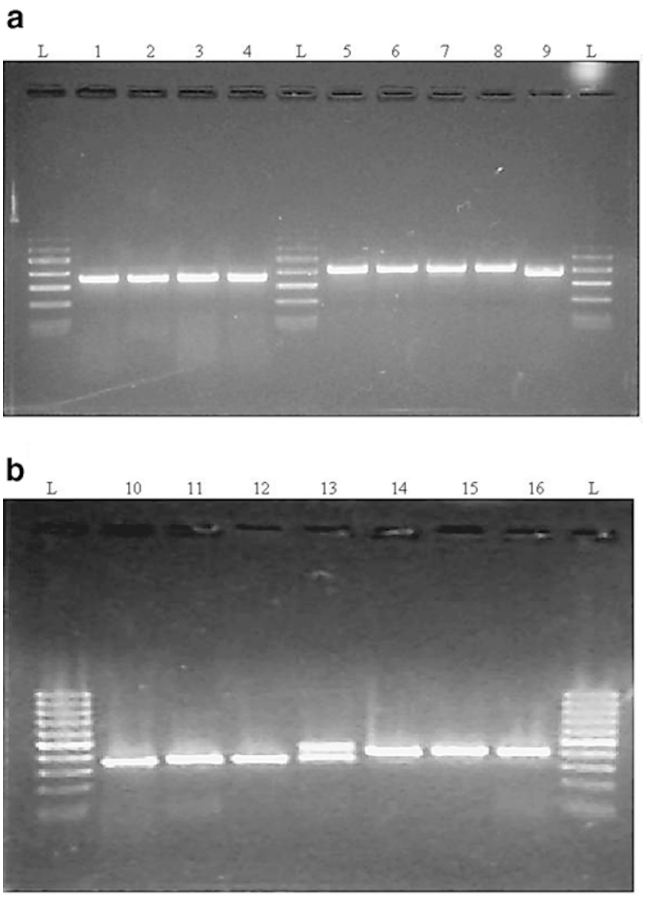

Figure 4 Gel electrophoresis photos of ITS-1 PCR amplifications. Lanes marked 'L' are DNA markers in 100 bp increments (100 to 1000 bp). (**a**) Comparison of *Cordulegaster bilineata* and *C. diastatops* ITS-1 products. Lane 1 – *C. bilineata* (Gallagher Fen, Ohio (OH)), Lane 2 – *C. bilineata* (Freeman Property, Michigan (MI)), Lane 3 – *C. bilineata* (Forest Glen, Illinois), Lane 4 – *C. bilineata* (Kiser Lake, OH), Lane 5 – *C. diastatops* (Barre Dam, Massachusetts (MA)), Lane 6 – *C. diastatops* (E. Branch Reservoir, OH), Lane 7 – *C. diastatops* (Shingleton, MI), Lane 8 – *C. diastatops* (The Glades, Maryland (MD)). Lane 9 is the ITS-1 product of a third species, *C. maculata*. Notice the ~50 bp size difference between *C. bilineata* and *C. diastatops*. (**b**) Comparison of problematic Wisconsin (WI) population. Lane 10 – *C. bilineata* (OH), Lane 11 – *C. bilineata* (Alabama), Lane 12 – Male 1 from Spur Lake, WI, Lane 13 – Male 2 from Spur Lake, WI, Lane 14 – Female from Spur Lake, WI, Lane 15 – *C. diastatops* (Pennsylvania), Lane 16 – *C. diastatops* (MA). Male 1 has an ITS-1 product of similar size to *C. bilineata*. The female specimen matches the ITS-1 product of *C. diastatops*. Male 2 appears to have ITS-1 products that match both *C. bilineata* and *C. diastatops*.

Ohio to Michigan) and show no sequence variation. The sequenced individuals of *C. diastatops* come from a similarly sized geographic area (Maryland, New York, New Jersey, and Massachusetts) and show only slight sequence variation (an extra GAGA near the region missing relative to *C. bilineata*).

This variation in *C. diastatops* near the 51 bp missing from *C. bilineata* may explain how this deletion could have occurred. The ITS-1 sequence upstream and downstream of the deletion is rich in A and G. The AG rich areas on both sides of the deletion could have led to unequal crossing over or could have caused slippage during replication that excised the 51 bp. Molecular drive would have spread the deletion to other copies of the

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Diastatops(MA)     1 CCGCTCGTGAACGAGATTCCGAGGTGGAAAAAGTGAGGGAGAGAGAGAGAGA-----TC
Diastatops(MD)     1 ..........................................................GAGA..
Bilineata          1 ..........................................T...----------...-------
Maculata           1 ........................T.....--------...----------T-------
Erronea            1 ..........................................T...G...G.G......GAGAGA

Diastatops(MA)    57 GCAGGCCGAGGCGGCGACTCCTCCTCCTCCGTGTGACGAG------------------GA
Diastatops(MD)    61 ..........................................------------------..
Bilineata         46 ------------------------------------------------------------..
Maculata          38 --------------------..................A---------------GA..
Erronea           61 .ATC..A.-.---------.....................GATCCCCGCCGAGTGCGG.G

Diastatops(MA)    99 GAAAGAGAGATGGAACGGAAAATGAACCAGCGTCGGCCGGGTACCTGCCGCCTCCCTCTT
Diastatops(MD)   103 ............................................................
Bilineata         48 ............................................................
Maculata          63 ..G.....T.........................G.............T.........
Erronea          111 .GG..........................................................

Diastatops(MA)   159 TCGAGACGGCGAGAGCGCGCGGGTTTTCAATGTCCTTCGCCCGGTCTTCGCAGGGGGTTG
Diastatops(MD)   163 ............................................................
Bilineata        108 ...........................................G................
Maculata         123 ......................A....................G................
Erronea          171 ...........................................G................

Diastatops(MA)   219 AGAAAATCCCGTCTGTCGCATTGCA-GAGGATGGATCCGGTCGGAGCTCCGAAACACGTT
Diastatops(MD)   223 ..........................-...........A.......................
Bilineata        168 ..........................-.......................T...........
Maculata         183 ..........................-............A.NT......T....CTG....
Erronea          231 .........................AN.........................CT.....

Diastatops(MA)   278 TCGGACCCGCTCAAAAAAA-TACAAAATTTGAAAAACATATCCTAG
Diastatops(MD)   282 ...................-..........................
Bilineata        227 ...................-..........................
Maculata         242 ...................-..........................
Erronea          291 ..................A...........................
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Figure 5 Alignment of ITS-1 sequences (5′–3′) of *Cordulegaster diastatops* (MA) ($n = 4$), *C. diastatops* (MD) ($n = 1$), *C. bilineata* ($n = 4$), *C. maculata* ($n = 1$), and *C. erronea* ($n = 1$). Notice AG rich areas before and after the 51 bp missing from *C. bilineata*.

gene (Dover, 1982). Alignment of the ITS-1 sequences of the four different species of *Cordulegaster* also shows that the most of the variation between all four taxa occurs in this particular portion of the ITS-1 and suggests that this region of the ITS-1 is quite variable.

The lone WI population may represent a problem for determining species status of these taxa, since different individuals within this population have ITS-1 products representative of both taxa. Under a strict interpretation of the biological species concept, the individual that appears to have both size ITS-1 bands would be strong evidence for hybridization between *C. bilineata* and *C. diastatops*, sinking *C. bilineata* as a separate species. This evidence for hybridization is quite tenuous, as it only includes three individuals with only one showing both ITS-1 bands. Hybridization between insect species, however, does not necessarily suggest high levels of gene flow between taxa as differences between species are maintained outside the hybrid zone (Jiggins *et al*, 1997; Willet *et al*, 1997). The presence of both bands could also be caused by incomplete homogenization of the deletion in the ITS-1 region throughout the genome.

Even if we assume that this evidence is sufficient to demonstrate interbreeding between *C. bilineata* and *C. diastatops*, several reasons exist to suggest that *C. bilineata* be considered a valid species. First, we have no way of knowing the fitness of the dual ITS band individual (although it did reach adulthood) or if it had the ability to produce viable offspring. Second, hybridization is known to occur between well-accepted, separate species of other insects, including crickets (Willet *et al*, 1997), butterflies (Jiggins *et al*, 1997), and damselflies (Leong and Hafernik, 1992). Male dragonflies are well known for their lack of discrimination when attempting copulation, as reports of copulation attempts with females of different species, genera, and even families are known to occur (Bick and Bick, 1981; Corbet, 1999). Finally, these three individuals from a single population simply do not represent enough data to support the idea that *C. bilineata* and *C. diastatops* readily hybridize. The combination of

both morphological and DNA sequence data is strong enough for the distinction of *C. bilineata* and *C. diastatops* as separate species to be maintained.

Unfortunately, we were unable to find morphological characters that will always conclusively distinguish between the two species. Color characters are used extensively in the original description of *C. bilineata* (Carle, 1983), but these characters are quite subjective. *Cordulegaster bilineata* is described as having a brown body with thinner thoracic stripes to that of the blacker *C. diastatops* (Carle, 1983). This distinction, however, is based mainly on museum specimens whose colors could be influenced by their age when captured, how they were preserved, and how long ago they were collected. We chose to eliminate color characters from our analysis because the body color was too difficult to quantify objectively and the thickness of the thoracic stripes was impossible to measure consistently due to the variable edges of these stripes. That color characters of this nature are too vague to be useful in taxonomy was evident in specimens of *C. bilineata* we collected from western MI. These were very dark and could be confused with *C. diastatops* based on color alone, although their ITS band and their EDW to CL ratio identified them as *C. bilineata*. Though imperfect, the ratio of EDW to CL seems to be the single best morphological character for determining *C. bilineata* from *C. diastatops*.

This study shows how morphological tools can be combined with DNA sequence data to address questions of species-level taxonomy. As DNA sequencing becomes more readily available, more studies could combine molecular and morphological techniques to address other taxonomic issues. Among *C. bilineata* and *C. diastatops*, anal appendage measurements should be predictive in identifying around 95% of individuals. Adding molecular methods should allow 100% correct identification between these two taxa. The ability to accurately distinguish between *C. bilineata* from *C. diastatops* is important for study of the distribution and habitat requirements. Although, neither is currently in danger of extinction, neither species is common and both are often known from only a few locations within a given state. The rarity of either species is probably related to their relatively specific habitat requirements of preferring small, forested seeps and streams (often found in bogs or fens, wetland habitats that are becoming increasingly sparse in the eastern United States). Future work with these two species should center on determining the nature of any hybridization or hybrid zone in the WI population and also on using molecular identifications as a tool for finding other more useful morphological characters for ease of distinction between these two similar, but separate species.

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