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Research article

Dietary Niche Partitioning of *Euphaea formosa* and *Matrona cyanoptera* (Odonata: Zygoptera) on the Basis of DNA Barcoding of Larval Feces

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Abstract

Odonate larvae are commonly considered opportunistic general predators in freshwater ecosystems. However, the dietary breadth of most odonate larvae in forest streams is still poorly documented. We characterized the prey species and estimated the level of dietary niche overlap of two damselflies, *Euphaea formosa* Hagen 1869 and *Matrona cyanoptera* Hämäläinen and Yeh, 2000 in a forest stream of central Taiwan on the basis of DNA barcoding of larval feces. A collection of 23 successfully identified *cytochrome c oxidase 1* (*CO1*) barcoding sequences suggested that the mayflies (Ephemeroptera), caddisflies (Trichoptera), and midges (Diptera) comprise the majority (43%, 6/14) of prey species consumed by *E. formosa* larvae, whereas the identified prey for *M. cyanoptera* were mainly zooplankton (56%, 5/9). Statistical analysis of dietary overlap indicated that these two species occupy different dietary niches (Pianka's index = 0.219). DNA barcoding analysis of damselfly larval feces was effective in detecting less sclerotized prey such as vertebrates (fish and frog) and small zooplankton. However, a moderately successful rate (<70%) of PCR amplification by universal *CO1* primers and a low percentage (<60%) of identifiable sequences in public databases indicate the limitations of naive DNA barcoding in fecal analysis.

Key words: Calopterygidae, dietary breadth, DNA barcoding, Euphaeidae, niche, Taiwan

Introduction

The Odonata—dragonflies and damselflies—contain numerous large species with conspicuous mating behaviors, making them one of the most popular research subjects in insect ecology and evolution (Córdoba-Aguilar 2008). They are often viewed as opportunistic general predators feeding on almost all available invertebrates and sometimes conspecifics (Corbet 1999, Robinson and Wellborn 1987). This generalization of trophic relationships between odonates and their prey is essential in studying community structures and ecological processes of freshwater ecosystems, including predator–prey interaction, niche partitioning, and the dynamics of food webs (Polis and Strong 1996, Kaartinen et al. 2010). However, a few studies have indicated that the diet of larval odonates varies according to habitat, season, larval size, and prey selection, making the assumption of generalist predators for larval odonates an oversimplification (Blois 1985, Robinson and Wellborn 1987, Dudgeon 1989a, Galbreath and Hendricks 1992, Khelifa et al. 2013). Adults of certain odonates were also found to be specialized predators on spiders and butterflies, or temporary specialists on honey bees (Corbet 1999).

The diets of larval odonates can be identified by examining the fecal pellets voided after consumption (Thompson 1978, Folsom and Collins 1984, Dudgeon 1989a, 1989b). The crushed body parts of prey from the feces can be compared with the morphological characteristics of potential prey species dwelling in the habitat. This method is often time intensive and largely lacks the ability to detect less sclerotized prey, except for a few diagnostic characteristics such as the radula of mollusks (Baker 1986, Corbet 1999). A library of body parts from potential prey species can facilitate morphological identification of fecal pellets. Immunological methods are also useful for detection of unsclerotized prey, especially the protozoans that are frequent in the diet of early instar larvae (Onyeka 1983, Sukhacheva 1996). The development of DNA barcoding using standardized genetic markers provides an alternative method to characterize the dietary breadth of insect predators (Hebert et al. 2003, 2004). The use of approximately 700-bp fragments of mitochondrial *CO1* gene was successful in dietary identification of many insect groups, for example, the gut contents of parasitoid wasps (Rougerie et al. 2011) and natural prey of water bugs (Gamboa et al. 2012). Molecular identification of prey species from the feces ("biodiversity

capsules") of generalist predators was recently proposed as a new tool for biodiversity and ecological assessment (Boyer et al. 2015). However, the effectiveness of DNA barcoding for characterizing larval odonate dietary breadth remains largely unknown.

Here, we conducted a molecular dietary analysis of two endemic damselfly species, *Euphaea formosa* Hagen 1869 (Euphaeidae) and *Matrona cyanoptera* Hämäläinen and Yeh 2000 (Calopterygidae), in a Taiwan forest stream. The aims of this study were to test the utility of DNA barcoding in characterizing the larval dietary breadth of *E. formosa* and *M. cyanoptera* and to determine the level of dietary overlap between them.

Materials and Methods

Damselflies and collection of fecal pellets

Larvae of *E. formosa* and *M. cyanoptera* were collected every two months between April 2010 and May 2011 (for a total of seven days), using nets and by hands, from three sites in the WuChen stream near LianHwaTz Forestry Research Station in central Taiwan (23°53′17″N, 120°53′50″E; 23°54′48″N, 120°53′44″E; 23°54′43″N, 120°53′17″E). The collected larvae were brought to the laboratory and kept alive individually in plastic boxes (diameter 7 cm, height 5 cm) filled with water and connected to an air pump. The larvae were allowed to expel feces for 48 hrs. Expelled fecal pellets were collected, immersed in 95% EtOH, and frozen at a –20 °C freezer. The head width of the larvae was measured microscopically as a proxy for body size (*E. formosa*: 3.33 ± 0.96 mm, n = 126; *M. cyanoptera*: 2.39 ± 0.76 mm, n = 154), and the larvae were then released back to their original sites. To examine the likelihood of amplifying endogenous damselfly DNA from the voided feces, one larva from each of the two damselfly species was fed with a larva of the Asian tiger mosquito (*Aedes albopictus*). Subsequently, the excreted fecal pellets with known prey identity were collected and analyzed.

DNA extraction and sequencing

Genomic DNA was extracted from the collected feces using FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corporation, Pingtung, Taiwan). The barcoding fragments of the *CO1* gene of fecal DNAs were amplified using published universal primers for metazoan invertebrates (LCO1490 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO2198 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′, Folmer et al. 1994), and for lepidopterans (LEP-F1 5′-ATT CAA CCA ATC ATA AAG ATA T-3′ and LEP-R1 5′-TAA ACT TCT GGA TGT CCA AAA A-3′, Hebert et al. 2004). Each PCR reaction contained a total volume of 50 µL, composed of 100–300 ng of template DNA, 0.4 µM of forward and reverse primers, 0.2 mM of dNTPs, and 0.04 unit of ProTaq polymerase (Protech Technology, Taiwan). The PCR profile was as follows: denaturing at 94 °C for 3 min, 35 cycles of amplification at 94 °C for 1 min, followed by annealing between 49.5 °C (LEP-F1 and LEP-R1) and 52 °C (LCO1490 and HCO2198) for 45 s and at 72 °C for 1 min, and an extension step at 72 °C for 10 min. The PCR products were gel-extracted using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) and either sequenced directly in both directions on an ABI PRISM 377 automatic sequencer (Perkin Elmer, USA) at the Mission Biotech of Taiwan, or cloned into the pCR 2.l-TOPO vector (Invitrogen) and then sequenced to differentiate among multiple possible PCR products obtained from a single feces sample. The DNA sequences from both directions were assembled and manually edited in EditSeq (DNASTAR Lasergene package, Madison, WI, USA). These sequences were translated into amino acid sequences using a mitochondrial genetic code of *Drosophila* in MacClade (Ver. 4.06, Maddison and Maddison 2000) to check for possible stop codons and indels caused by ambiguous sequencing, and then deposited in GenBank (accession numbers listed in Appendix 1).

Sequence identification and niche overlap index

The edited *CO1* sequences were searched through BLAST in the nucleotide collection database (nr/nt) of NCBI (http://www.ncbi.nlm.nih.gov) in September of 2015. The searches were conducted using BLASTN program (Ver. 2.2.27, Morgulis et al. 2008), which is optimized for highly similar sequences (Megablast) with parameters of the alignment algorithm set to the default values of 10 for expected number of chance matches, and 1/−2 for match(reward)/mismatch(penalty) ratio, and linear gap costs. The sequences that yielded the best alignment (the highest maximum score) to all or a part of the query sequence were selected for candidate species identification. The sequences were also compared against a *CO1* barcode database available in the Barcode of Life Data (BOLD, Ver. 3) Systems (Ratnasingham and Hebert 2007). The sequence matching followed both a linear search for the nearest neighbors from global alignment and a neighbor-joining tree reconstructed from 100 nearest neighboring taxa in the BOLD Identification System. Sequences showing less than 1% and 3% divergence to the reference sequences without a close match to any other invertebrates were assigned to a species and genus, respectively. In the absence of a close match because of incomplete taxonomic coverage in the reference database, higher-level identifications were made by examining the clustering relationship of query sequences within the neighbor-joining tree and the known taxonomy of neighboring sequences as indexed in BOLD.

Niche overlap between the diets of the two damselfly species were calculated using Pianka's (1973) index in EcoSimR (Gotelli and Ellison 2013), which assesses the degree of overlap between the two species along one dimension of dietary niche. The value of this index varies from 0 (no overlap) to 1 (complete overlap). The significance of niche overlap was obtained by resampling the dataset 10,000 times by using the RA3 randomization algorithm (retaining niche breadth of each species but randomizing utilized resource states, Winemiller and Pianka 1990).

Results

Success rate of PCR amplification

A total of 52 and 104 fecal pellets were collected from 126 *E. formosa* and 155 *M. cyanoptera* larvae, respectively (Appendix 1). Using the barcoding primers for metazoan invertebrates (LCO1490 and HCO2198), the percentage of successful PCR amplification was higher in fecal DNA samples of *E. formosa* (37%, 19/52) than those of *M. cyanoptera* (24%, 25/104). When the lepidopteran primer set (LEP-F1 and LEP-R1) was applied to the remaining DNA samples unamplified by LCO1490 and HCO2198, the success rate was again higher in *E. formosa* (52%, 17/33) than *M. cyanoptera* (39%, 31/79). Overall, the success rates of PCR amplification of *CO1* barcoding region of all fecal samples were 69% (36/52) and 54% (56/104) for *E. formosa* and *M. cyanoptera*, respectively. Unambiguous *CO1* sequences of mostly over 450 bp (ranging from 243 to 708 bps) were obtained from 25 *E. formosa* (69% of 52) and 26 *M. cyanoptera* (54% of 104) feces samples (Appendix 1), creating the possibility of their taxonomic identification in reference databases. Cloning and sequencing PCR products of individual feces suggested that

Table 1. List of prey taxa identified in the larval feces of *Euphaea formosa* and *Matrona cyanoptera* using *CO1* barcoding sequences.

Prey	Common Name	Order/Higher Taxa	Family	Species	*E. formosa*	*M. cyanoptera*	Specimen Code (Head width, mm)
Insects	Mayfly	Ephemeroptera	Baetidae	*Labiobaetis atrebatinus*	2	0	E008(4.02), E013(3.15)
	Caddisfly	Trichoptera	Philopotamidae	*Chimarra* sp.	1	0	E015(2.28)
	Fly & Midge	Diptera	Chironomidae	*Phaenopsectra flavipes*	0	1	M020(1.67)
			Calliphoridae	*Compsomyiops callipes*	1	0	E048(4.49)
			Culicidae	*Aedes vexans*	1	0	E019(4.6)
			Muscidae	*Haematobosca alcis*	1	0	E030(3.61)
			Drosophilidae	*Phortica* sp.	0	1	M056(2.58)
	Flea	Siphonaptera			2	0	E081(2.59), E126(4.99)
	Bee, Ant & Wasp	Hymenoptera			0	1	M103(2.71)
Invertebrates	Zooplankton				1	5	E059(1.63), M001(2.69), M013(2.1) M031(1.61), M035(3.78), M046(3.58)
	Rotifer	Bdelloidea	Philodinidae	*Macrotrachela sp.*	1	0	E058(2.64)
	Water Flea				1	0	E101(4.32)
		Cyclopoida	Cyclopidae	*Mesocyclops longisetus*	1	0	E112(5.04)
	Mollusks	Bivalvia			0	1	M051(1.3)
Vertebrates	Ray-Finned Fish	Clupeiformes	Engraulidae	*Engraulis japonicus*	1	0	E037(2.99)
	Frog	Anura	Leptodactylidae	*Adenomera ajurauna*	1	0	E073(3.78)
Total					14	9	

each fecal sample contained a single prey species. The results of sequencing fecal DNA with known prey of Asian tiger mosquito confirmed the low levels of contamination from the damselfly's own DNA.

Prey identification and niche overlap

Comparison to NCBI and BOLD databases resulted in identification of 59% (30/51) of the obtained barcoding sequences to potential prey species, genus, or higher taxa (Table 1). The remaining 41% (21/51) of the sequences were either not identifiable or revealed sequence similarity to bacteria and water molds, which are unlikely prey of damselfly larvae and probably contaminants from prey body surfaces (Appendix 1). A total of 23 barcoding sequences were identified to genus or species (Table 1). Approximately one-half of all identified prey were insects (48%, 11/23), including mayflies (Ephemeroptera), caddisflies (Trichoptera), midges (Diptera), fleas (Siphonaptera), and hymenopterans (Hymenoptera). Cannibalism and intra-odonate predation were not detected. The majority of non-insect prey items were invertebrates (39%, 9/23), consisting of zooplankton, rotifer (Bdelloidea), water flea (Cyclopoida) and mollusks (Bivalvia). Ray-finned fish (Clupeiformes) and frog (Anura) were the only two vertebrate prey identified. Mayfly, flea, and zooplankton were identified twice or more while other prey were noted only once each. Among all identified higher taxa, only flies and zooplankton were preys shared between *E. formosa* and *M. cyanoptera*. Mayfly, caddisfly, flea, rotifer, water flea, ray-finned fish, and frog were exclusively consumed by *E. formosa*, whereas the hymenopterans and mollusks were prey specific to *M. cyanoptera*. The observed value of between-species niche overlap (Pianka's index = 0.219) was significantly lower than expected (0.347 ± 0.018; $P = 0.044$), suggesting that these prey species are associated with different regions of dietary niche space.

Discussion

Our data indicate that *E. formosa* and *M. cyanoptera* larvae tend to occupy different dietary niches in the stream under study in central Taiwan. The observed value of between-species niche overlap (Pianka's index = 0.219) is lower than that of interspecific niche overlap between congeneric *E. decorata* and two gomphid dragonfly species, *Heliogomphus scorpio* and *Onychogomphus sinicus* (Gomphidae) (Pianka's index = 0.44 to 0.55, Dudgeon 1989a). Mayfly (Ephemeroptera), caddisfly (Trichoptera), and midge (Diptera) comprised the majority (43%, 6/14) of prey species consumed by *E. formosa* larvae. These three insect groups are consistent with the prey taxa most frequently found in the diet of congeneric *E. decorata* in a Hong Kong forest stream (Dudgeon 1989b). The prey identified for *M. cyanoptera* was mainly zooplankton (56%, 5/9). The low occurrence of most of prey species in the diet of both species may reflect the opportunistic predatory feeding behavior of the damselfly larvae, or the effect of low sampling size (successfully identifiable barcoding sequences). Primarily terrestrial insects, including a flea (Siphonaptera) and a hymenopteran, were identified as prey items for the two damselfly larvae; this was unexpected because these are rarely reported as odonate prey (reviewed in Corbet 1999). Nevertheless, the molecular identification of these two prey species may have been incorrect through sequence similarity to members of these insect orders or as a result of incomplete taxonomic coverage of the reference databases in NCBI and BOLD. Although our analysis suggested that the ecological niches of *E. formosa* and *M. cyanoptera* were separated by dietary dimensions, the limited number of identifiable prey based on DNA barcoding ($n = 23$) renders this conclusion preliminary. In this study, the number of observed, shared prey species between *E. formosa* and *M. cyanoptera* is likely an underestimate of the actual number of shared prey, especially rare species.

Habitat, food, and time are considered the three most important ecological niche axes, and niche partition among species generally occurs along the first two axes (Schoener 1974, Crowley and Johnson 1982). Our findings support this assertion and imply that dietary divergence is an essential niche dimension for larval damselfly. The underlying mechanism for dietary niche partitioning between these two damselflies is currently unknown. Studies showed that the difference in microhabitats of odonate species can increase

the likelihood of spatial separation (Dudgeon 1989a, Khelifa et al. 2013); thus, microhabitat specialization may subsequently result in dietary divergence. During the daytime, *Euphaea formosa* larvae rest chiefly on the underside of raised and submerged rocks in strongly flowing currents (Hayashi 1990, Huang and Lin 2011), whereas those of *M. cyanoptera* often perch on tree roots, branches, and stems in unshaded slow-moving streams (Matsuki and Lien 1978, Hämäläinen and Yeh 2000). These field observations suggest that microhabitat specialization appears to be critical for spatial separation of dietary niche dimension in *E. formosa* and *M. cyanoptera*. Among the three major ecological niche axes, temporal activity seems to be the least important dimension for these two species. These two damselflies have similar life cycles (univoltine, continuous larval growth throughout the year, and adult emergence from February to November) (Matsuki and Lien 1978, Hayashi 1990), greatly reducing the likelihood of temporal segregation of ecological niches. In addition to microhabitat specialization as a possible explanation for dietary partitioning between *E. formosa* and *M. cyanoptera*, species-specific traits including feeding morphology (Pritchard 1964, 1965), foraging strategies (Folsom and Collins 1984, Hirvonen and Ranta 1996), and prey selection (Galbreath and Hendricks 1992) can also lead to dietary selection and partitioning among coexisting odonate larvae. The potential underlying mechanistic explanation for dietary niche partitioning between *E. formosa* and *M. cyanoptera* requires further investigation.

The important findings of this study are the identification of two vertebrates (fish and frog) consumed by *E. formosa*, and the zooplankton and soft-bodied mollusks (Bivalvia) as prey items for *M. cyanoptera*. This demonstrates the effectiveness of DNA barcoding for detecting less sclerotized prey species and freshwater zooplankton that are frequently unrecognizable through direct examination of larval feces. Small or soft-bodied prey may be more frequent in the diet of odonate larvae than previously recognized and therefore could constitute a significant proportion of prey items underestimated by morphological characterization of fecal samples. Nevertheless, our results for an overall moderate success rates ($< 70\%$) of PCR amplification for *CO1* barcoding sequences indicate the limitation of species identification for fecal samples based on DNA barcoding. We suggest the development of taxon-specific *CO1* barcoding primers, similar to the approach by Zeale et al. (2011), which would be helpful in increasing successful PCR amplification of all potential prey species dwelling in a particular habitat. On the basis of a global BLAST search in NCBI and BOLD databases, our study showed that less than 60% of the DNA barcode sequences were identifiable to probable prey items. This finding demonstrates the need for a regional *CO1* barcoding reference database of forest stream fauna based on known species identification to facilitate a more comprehensive characterization of the dietary breadth of the damselfly.

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