SPECIES RECOGNITION AND PATTERNS OF POPULATION VARIATION IN THE REPRODUCTIVE STRUCTURES OF A DAMSELFLY GENUS

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The selection pressures imposed by mate choice for species identity should impose strong stabilizing selection on traits that confer species identity to mates. Thus, we expect that such traits should show nonoverlapping distributions among closely related species, but show little to no variance among populations within a species. We tested these predictions by comparing levels of population differentiation in the sizes and shapes of male cerci (i.e., the clasper structures used for species identity during mating) of six *Enallagma* damselfly species. Cerci shapes were nonoverlapping among *Enallagma* species, and five of six *Enallagma* species showed no population variation across their entire species ranges. In contrast, cerci sizes overlapped among species and varied substantially among populations within species. These results, taken with previous studies, suggest that cerci shape is a primary feature used in species recognition used to discriminate conspecific from heterospecifics during mating.

KEY WORDS: *Enallagma*, mate choice, Odonata, population variation, species recognition.

The decisions that individuals make about who to accept and who to reject as mates define species boundaries of sexual species and shape the potential for sexual selection within populations. Animals typically base mating decisions on the phenotypes of potential mates, and so these decisions can impose strong selection on these phenotypes because they shape the fitnesses of the chooser, the chosen, and the unchosen (Andersson 1994). However, these different types of choices imply that characters signaling species identity and characters signaling mate quality are under contrasting selection pressures, because individuals have contrasting goals for these decisions when choosing among potential mates (Pfennig 1998).

In assessing the species identity of a potential mate, an individual is trying to minimize the probability of mating with heterospecifics that will produce few or no offspring or offspring with low fitness (i.e., Dobzhansky 1940; Butlin 1987; Howard 1993; Servedio 2001). As a result, individuals may advertise their species identity to potential mates, and these traits should be under strong stabilizing selection via mate choice for species identity: any deviation from the norm should be strongly disfavored (Templeton 1979; Paterson 1993). Moreover, these traits should be unambiguous signals of species identity (McPeek and Gavrilets 2006). Thus, traits conferring species identity should (1) differ significantly among species to minimize mistakes in mate identity, and (2) should vary little among populations across a species' range because strong stabilizing selection for the same trait values in each population should inhibit population differentiation (Paterson 1993; Butlin 1995). Many different types of traits are thought

to confer species identity in different taxa, including breeding coloration, calls and songs, pheromones, the shapes of morphological structures involved in breeding, behavioral displays, and genetic compatibility signals in gametes (e.g., Searcy and Nowicki 2000; Shaw 2000; Swanson and Vacquier 2002; Gerhardt 2005). These traits are thought to signal species identity because experimentally altering these traits greatly decreases the likelihood of an individual's being chosen as a mate (e.g., Robertson and Paterson 1982; Phelps et al. 2006), and because of their usefulness in taxonomic classification (Eberhard 1985; Huber 2003).

In contrast, most other traits need not differ among species, and myriad other processes may generate significant population variation within species. Traits may differ among populations because of phenotypic plasticity in response to environmental conditions (e.g., populations may differ in average body size because of differing resource levels available to individuals in those populations), or because of evolved responses to spatially varying selection regimes (e.g., populations have different genetic predispositions for body size that are the result of differences in past selection). Even mate choice among conspecifics does not necessarily favor species differentiation and homogeneity of traits across populations within species. Individuals choose among conspecific mating partners for many different reasons (e.g., Fisherian runaway processes [Grafen 1990; Andersson 1994], to obtain better genetic parents for their offspring [Møller and Alatalo 1999; Neff and Pitcher 2005], sensory biases [Endler 1992; Fuller et al. 2005]), and the traits used to discriminate among conspecific mates by one species need not interfere with the conspecific mating choices of another species (see Pfennig 1998 for a full discussion of these issues). Also, the form and strength of sexual selection may differ substantially among populations (Endler 1977; Simmons et al. 2001; Kwiatkowski and Sullivan 2002; Punzalan et al. 2010). Differences in environmental conditions may also favor different trait values because of sensory biases in mate choice (Endler 1992; Boughman 2001; Fuller et al. 2005). In addition, trait variation among populations is expected with population variation in any countervailing natural selection pressures on the trait in question, other traits to which this trait is linked through trade-offs, or any costs to mate choice (e.g., Lande 1981, 1982; Kirkpatrick 1982; Day 2000). Any differences in the underlying genetic architecture of these traits (heritabilities and genetic correlations with other traits) can also influence how far and in what directions sexual selection can alter the trait in any given population (Lande 1981, 1982; Kirkpatrick 1982).

In this article, we test the predictions about species identity traits by examining interspecific and intraspecific levels of differentiation in traits involved in mate choice among species of *Enallagma* damselflies (Zygoptera: Coenagrionidae) (Fig. 1). In *Enallagma*, females identify males to species by the tactile cues they receive when males clasp them with their cerci, the
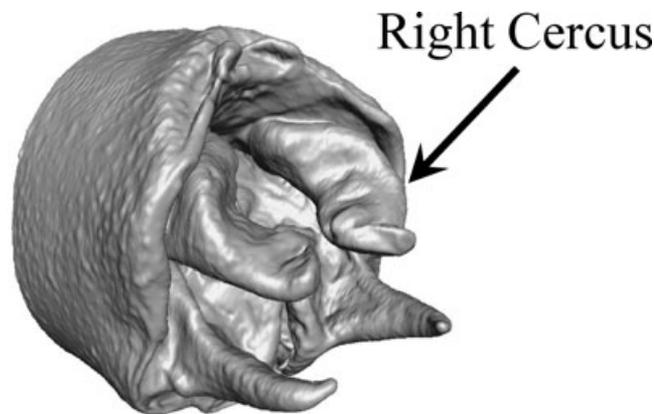
Right Cercus

Enallagma hageni

Figure 1. Model from a computer tomographic scan of the 10th abdominal segment of *Enallagma hageni* showing the four male abdominal appendages used to grasp females during mating. The right cercus of the male is identified.

terminal abdominal appendages that males use to hold females while they mate (Krieger and Krieger-Loibl 1958; Paulson 1974; Robertson and Paterson 1982; Tennessen 1982). *Enallagma* females have characteristic dorsal prothoracic plates where the male cerci make contact while clasping (Westfall and May 2006); presumably, females use the tactile cues they receive on these plates when grasped by males to determine the male species identity. Females reject heterospecific males shortly after being clasped (Paulson 1974; Fincke et al. 2007); and when cerci are experimentally altered, females reject conspecific males (Robertson and Paterson 1982). The shapes of male cerci and female plates differ substantially among species (e.g., McPeek et al. 2008, 2009), and the shapes of the male and female structures have evolved in a correlated fashion across the history of the genus (McPeek et al. 2009).

Specifically, we evaluated the degree of species overlap and population differentiation in *Enallagma* cerci size and shape. Because cerci shape is thought to confer species identity to potential mates, we predicted that *Enallagma* species should be nonoverlapping in cerci shape, but that populations within *Enallagma* species should not differ in cerci shape. In contrast, we predicted that *Enallagma* cerci size would overlap among species, and vary among populations, because cerci size is probably correlated with overall body size, and many ecological factors (e.g., resource levels) influencing overall body size vary among lakes inhabited by *Enallagma* (e.g., Siepielski et al. 2010 and in review).

Materials and Methods

We quantified size and shape variation in male cerci for six *Enallagma* species from populations scattered across their ranges.

Table 1. Locations of *Enallagma* populations sampled for the study and sample sizes for the cerci morphology (N-Cerci) studies.

Species	N	Population (Latitude/Longitude)
Enallagma annexum	8	Tabor Lake, Prince George, British Columbia (N53°55.03′,W122°32.55′)
	1	Ketchmark #1, Brooks, Alberta (N53°35.03′,W111°53.21′)
	1	Chickakoo Lake, Stony Plane, Alberta (N53°32.03′,W114°02.51′)
	12	Pond Lab Reservoir, Hickory Corners, Michigan (N42°24.58′,W85°23.65′)
	4	Lily Pond, Kancamagus Pass, New Hampshire (N44°1.10′,W71°27.17′)
Enallagma aspersum	2	Warren Pond, Belle Marsh, Road, South Berwick, Maine (N43°13.71′,W70°43.66′)
	6	Moose Pond, H Road, North Shapleigh, Maine (N43°35.30′,W70°56.04′)
	3	Pond Lab Reservoir, Hickory Corners, Michigan (N42°24.58′,W85°23.65′)
	9	farm pond, Georges Reserve, Pinchney, Michigan (N42°27.91′,W84°0.21′)
	5	Rabinowicz Pond, Elm Road, Etna, New Hampshire (N43°42.67′,W72°12.74′)
	6	Long Pond, Block Island, Rhode Island (N41°9.60′,W71°36.10′)
Enallagma boreale	8	Little Cranberry Lake, Valemount, British Columbia (N52°48.00′,W119°15.06′)
	4	Blanche Lake, Medicine Lake Highlands, Siskiyou Co., California (N41°33.40′,W121°34.26′)
	7	Gaspisie Site 1, Sur # 132, Cloridorme, Quebec (N49°11.10′,W64°51.20′)
	5	Sylvester's Pond, Norwich, Vermont ((N43°43.85′,W72°18.67′)
Enallagma geminatum	12	Uncas Lake, Lyme, Connecticut (N41°22.49′,W72°18.94′)
	10	Scituate Pond, Scituate, Maine (N43°10.59′,W70°40.58′)
	12	Perley Pond, Sandy Beach Road, North Sebago, Maine (N43°54.04′,W70°40.12′)
	11	Holly Lake, Dennisville, New Jersey (N39°11.89′,W74°50.37′)
	3	Lake Kelso, South Newbury, Ohio (N41°26.66′,W81°10.48′)
	4	Martin's Lake, Sandhills National Wildlife Refuge, McBee, South Carolina (N34°33.28′,W80°12.99′)
Enallagma hageni	4	Arrowhead Lake, Viola Township, Lakeview, Iowa (N42°17.72′,W95°2.96′)
	17	Lovewell Pond, Fryeburg, Maine (N43° 59.99′,W70° 56.14′)
	9	Deep Lake, Hastings, Michigan (N42°35.30′, W85°27.43′)
	5	Three Lakes II, Richland, Michigan (N42°20.95′, W85°27.78′)
	6	Upper St. Croix, Solon Springs, Wisconsin (N46°22.95′, W91°46.87′)
Enallagma recurvatum	10	Snow Pond, Truro Township, Massachusetts (N41°58.19′,W70°1.66′)
	12	Makepeace Lake, Makepeace Wildlife Management Area, Atlantic County, New Jersey (N39°32.54′,W74°44.64′)

Table 1 lists the locations and sample sizes. Populations for some species were as much as 3800 km apart. The six *Enallagma* species are members of the recent radiation in North America (Turgeon et al. 2005). Where their ranges overlap, *E. hageni*, *E. geminatum*, and *E. recurvatum* are found breeding together at ponds and lakes with fish, and *E. aspersum*, *E. boreale*, and *E. annexum* are found breeding together at ponds and lakes where dragonflies are the top predators (McPeek 1990, 1998).

After collection, males were stored in a −80°C freezer until used in this analysis. For analysis, males were first placed in a drying oven at 40°C for 24 h to dry all internal tissues: this drying does not change the external morphology of the cerci, but makes digitizing structures from the scans easier. The abdomen of each male was then removed, mounted on a brass stem with dialysis tubing and modeling clay, and scanned using computer tomography (CT) technology in a SkyScan 1172 high-resolution micro-CT scanner (SkyScan®, Kontich, Belgium). CT scans were made at a pixel resolution of 2.5 μm (i.e., voxel resolution of 15.6 μm³) through 180°, with a rotation step of 0.7°/frame, and averaging three frames. CT scans were converted to stacks of digital image slices using NRecon version 1.4.4 software (SkyScan).

The right cercus of each male was then segmented from the resulting digital image stack and initial processing was performed using Amira® version 5.2.2 software (Visage Imaging Inc., Andover, MA). The left and right cerci are mirror images of one another (McPeek et al. 2008, 2009), and so analyses were only performed on the right cercus. All voxels associated with each cercus were first identified using the editing and labeling tools. A high resolution triangular mesh surface model of each cercus was then constructed. For computational purposes, each triangular mesh surface model was reduced to have 20,000 triangles with 10,002 vertices. The positions of seven landmarks were then

identified on each cercus using Amira. These landmarks were only used to spatially register cerci models to one another.

We first calculated and recorded the centroid size of each cercus from the original triangular mesh surface. We then standardized each to have a centroid size of 1 to prevent confounding size and shape variation, and used spherical harmonics analyses of the resulting size-standardized triangular mesh surfaces to quantify cercus shape. Here we present a brief description of the spherical harmonic analysis; see McPeek et al. (2008) and Shen et al. (2009) for fuller descriptions of these algorithms and their applications to biological shapes. Spherical harmonics, an extension of the classic Fourier transform, represent a 3-dimensional (3D) shape in terms of a sum of 3D sines and cosines on a sphere (Brechbühler et al. 1995; Ritchie and Kemp 1999; Shen et al. 2007). The result is a set of complex coefficients that quantify the contributions of different spatial frequencies to the shape. These coefficients can be used to reconstruct the original shape, compute the distance between two shapes, and morph from one shape to another (McPeek et al. 2008, 2009; Shen et al. 2009). The coefficients are estimated by solving a series of linear equations using standard least squares estimation (Shen et al. 2009). Greater detail of the structure can be modeled by including higher frequency components. Cerci were modeled to degree $l = 18$, which produces $361 (= [l + 1]^2)$ coefficients to represent each of the three spatial dimensions, for a total of 1083 complex-valued coefficients. These algorithms were coded and run using Matlab version 7.8.0 (The Mathworks Inc., Natick, MA); this code is available at http://www.enallagma.com/SPHARM.php.

The resulting spherical harmonic coefficients provide a quantitative and orthogonal coordinate system to ordinate species based on shape (once standardized by centroid size). We tested for species and population differences in cerci shape within species using nonparametric multivariate analysis of variance (npMANOVA) (Anderson 2001). In simple univariate analyses of variance, sums of squares are constructed on the distances of data points to various means within the dataset (e.g., cell means and the grand mean) (Kutner et al. 2005). In like fashion, npMANOVA constructs sums of squares based on the distances of data points to the appropriate centroids for the experimental design being analyzed, but these sums of squares are constructed directly from these distances and not from the variance–covariance matrix among the response variables (Anderson 2001). Because the spherical harmonics coefficients form a quantitative and orthogonal basis for quantifying shape, we used Euclidian distances in calculating the npMANOVAs and constructed exact F-ratios for hypotheses (randomization tests gave qualitatively identical results) (Anderson 2001). We assume that both species and populations within species are random factors in all analyses. Also, because the hypotheses predict specific relationships for phenotypic distributions within and among species, and not about the

Table 2. The total variance in cerci shape among all the individuals, and the amount of variation within each species when considered separately.

	Total Variance
All *Enallagma* species	5.86×10^{-5}
E. annexum	9.83×10^{-6}
E. aspersum	1.47×10^{-5}
E. boreale	7.87×10^{-6}
E. geminatum	8.80×10^{-6}
E. hageni	9.57×10^{-6}
E. recurvatum	9.72×10^{-6}

evolutionary history of these distributions, we apply no evolutionary corrections to the phenotypes (for such analyses of cerci evolutionary shape variation, see McPeek et al. 2008, 2009). We also used principal components (PC) analyses to visualize the major axes of variation of cerci shape (Morrison 2004). PCs were extracted from the covariance matrix of spherical harmonic coefficients using the princomp procedure of Matlab.

Results

Figures 2A–G show representative right cerci of the six *Enallagma* species included in this study. The total amount of shape variation among all individuals of all species in the analysis was 5.86×10^{-5} (after standardizing each to a centroid size of 1), which was substantially greater than the total shape variation within each species (Table 2). This was because *Enallagma* species formed nonoverlapping clusters in cerci shape space (Fig. 3). Because of this, we identified substantial heterogeneity in cerci shape among species (npMANOVA, $F_{5,189} = 189.08$, $P < 0.001$; note that we interpret species as a random factor in this analysis).

In contrast to species differences, five of the six *Enallagma* species showed no heterogeneity in cerci shape among the populations (npMANOVAs: *E. aspersum* $F_{5,25} = 0.96$, $P > 0.45$; *E. boreale* $F_{3,20} = 1.53$, $P > 0.20$; *E. geminatum* $F_{5,46} = 1.23$, $P > 0.30$; and *E. hageni* $F_{4,36} = 1.26$, $P > 0.30$; *E. recurvatum* $F_{1,19} = 1.68$, $P > 0.20$), despite the fact that the population sampling covered huge areas of each species' range (e.g., the most distant populations for each were: *E. aspersum* 1180 km, *E. boreale* 3730 km, *E. geminatum* 1260 km, *E. hageni* 1950 km, *E. recurvatum* 480 km). *E. annexum* was the only species to show any population variation, and it had large and nonoverlapping differences among populations across its range (npMANOVA: $F_{4,21} = 4.27$, $P < 0.05$). Western *E. annexum* populations in British Columbia and Alberta were completely separated in cerci shape space from populations in Michigan and New Hampshire (Fig. 4). These populations differed in the size of the tubercle in the center of the dorsal surface (cf. Figs. 2F,G).
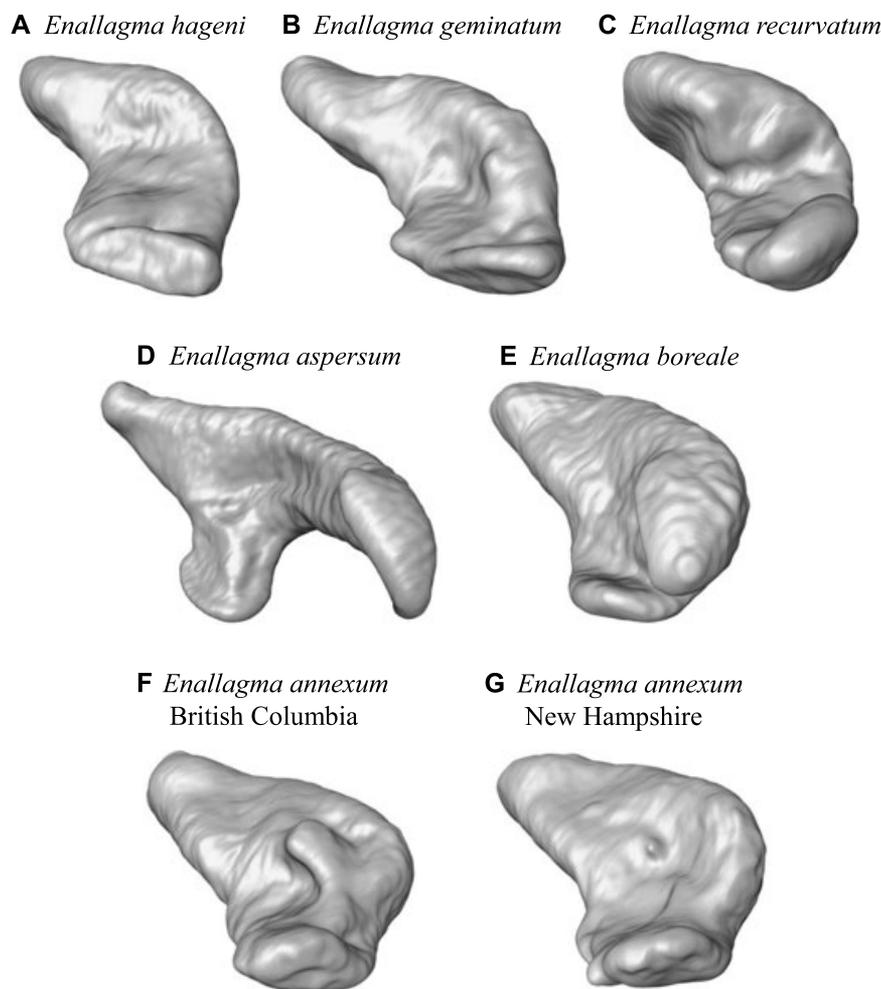
A *Enallagma hageni*   **B** *Enallagma geminatum*   **C** *Enallagma recurvatum*

D *Enallagma aspersum*   **E** *Enallagma boreale*

F *Enallagma annexum*
British Columbia

G *Enallagma annexum*
New Hampshire

Figure 2. Model representations of the male right cercus generated from computer tomography scans for the six *Enallagma* species (panels A–G) included in this study. Each cercus is viewed from the posterior, and the dorsal surface is visible (nearly identical orientation as in Fig. 1). Cerci for *E. annexum* males are shown from (F) British Columbia and (G) New Hampshire.

Unlike cercus shape, cercus size overlapped across species (Fig. 3E) and was highly variable among populations within most species (ANOVAs: *E. annexum* $F_{4,21} = 14.94$, $P < 0.001$; *E. aspersum* $F_{5,25} = 14.46$, $P < 0.001$; *E. boreale* $F_{3,20} = 2.25$, $P > 0.10$; *E. geminatum* $F_{5,46} = 3.62$, $P < 0.01$; and *E. hageni* $F_{4,36} = 8.44$, $P < 0.001$; *E. recurvatum* $F_{1,19} = 4.20$, $P = 0.055$). Because cercus size is correlated with sizes of other body parts (e.g., the correlation between cercus centroid size and abdomen length and between cercus centroid size and forewing length in *E. geminatum* were both $r_{31} = 0.72$, $P < 0.01$), this population-level heterogeneity almost certainly reflects differences in overall body size (see McPeek et al. 2008 for similar correlations in *E. hageni*).

Discussion

These results for *Enallagma* cerci shape confirm our predictions for traits involved in species identity. *Enallagma* species form

nonoverlapping clusters in cerci shape space, and so males can be unambiguously identified to species by females. The shapes of *Enallagma* cerci also did not vary among populations scattered across the entire ranges of five of the six species. *Enallagma* males are promiscuous, nonterritorial breeders that wait at the edges of ponds for females. Males of multiple *Enallagma* species can be found at a pond on any particular day during the breeding season: we have recorded up to six species simultaneously breeding at individual lakes (including the species included in this study if they are found at that lake type) in many areas of eastern North America (M. A. McPeek, unpublished data). In contrast, *Enallagma* females spend most of their time away from ponds foraging to acquire energy to produce eggs (Fincke 1982). Females only come to ponds to breed and oviposit. When a female arrives at a pond, males of many species attempt to grasp her with their cerci to initiate breeding. Females reject heterospecific males, which have incorrect cercal morphologies, presumably because they receive the wrong tactile cues from the contact and forces
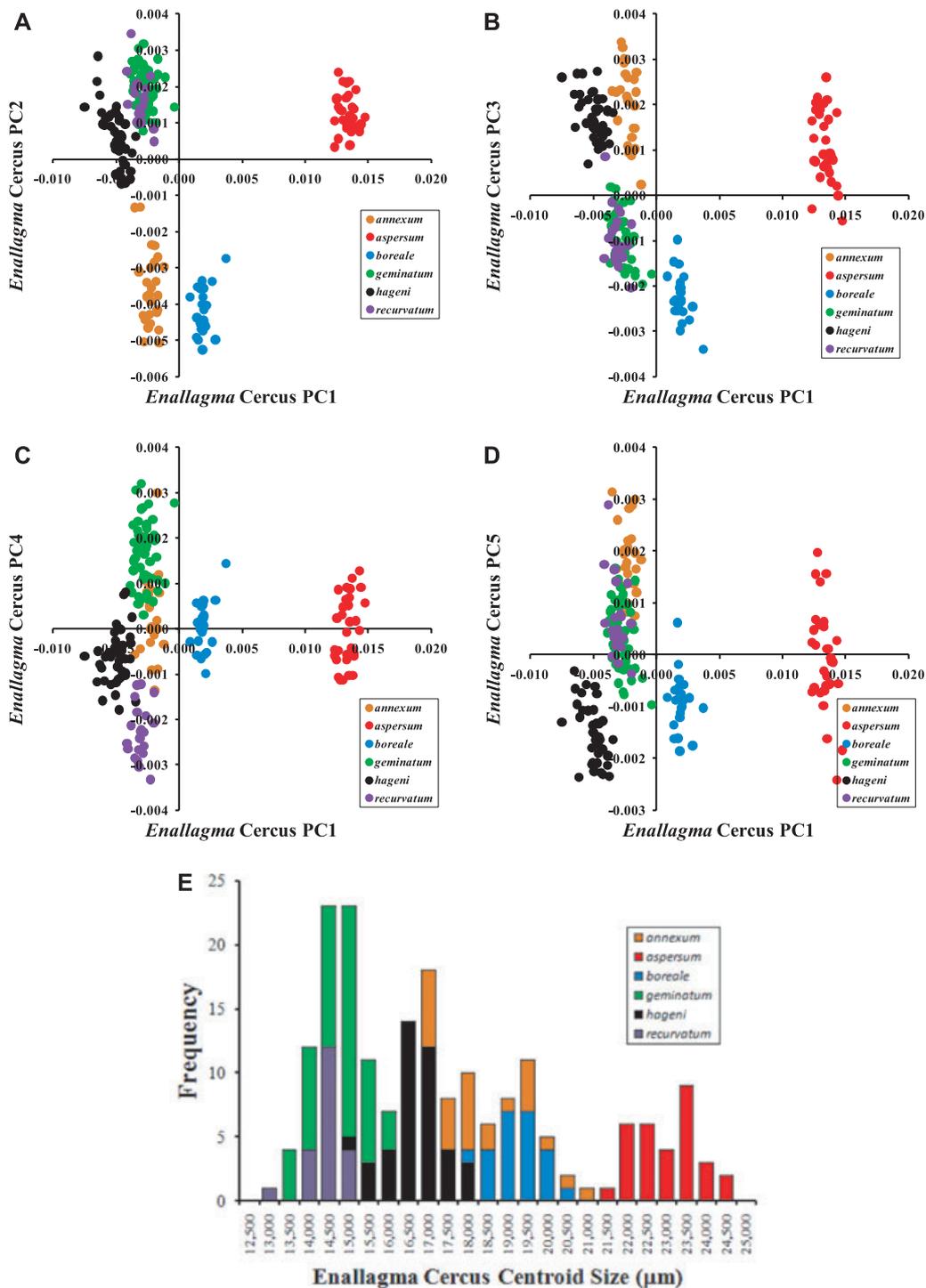
Figure 3. The five-dimensional ordination of cerci shape in principal components space of all *Enallagma* cerci included in this study and cerci centroid size variation. Panels A–D display the various 2D perspectives of the positions of the six species relative to one another in the spherical harmonics shape space. Each symbol represents an individual, and symbols of different colors identify different species (see legend). Panel E presents a frequency histogram of the centroid size variation of cerci among the six species.

imparted on their thoracic plates when grasped by the cerci of heterspecific males (Paulson 1974; Robertson and Paterson 1982; Tennessen 1982; Fincke et al. 2007). Supporting this interpretation is the fact that females refuse to mate with conspecific males

that have experimentally altered cerci (Robertson and Paterson 1982).

Only *E. annexum* showed significant variation in cerci shape across its range. Eastern and western populations differed in
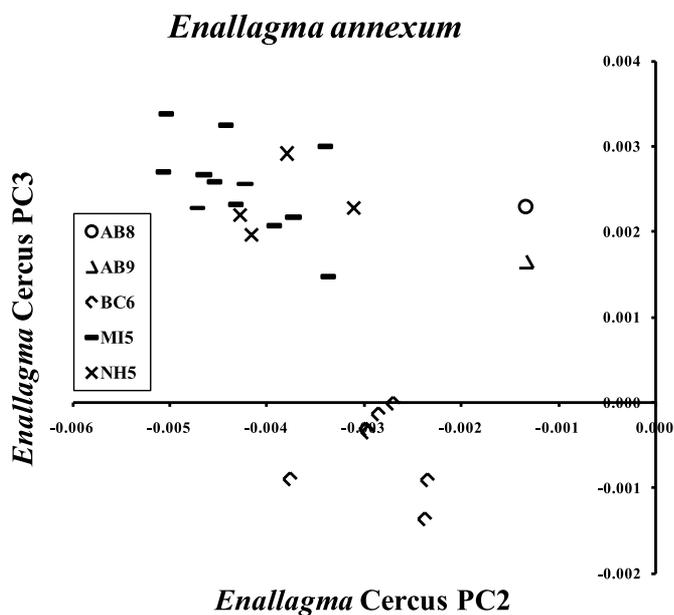
Enallagma annexum

Figure 4. The ordination of populations of individuals for *E. an-nexum* in the principal components extracted from the full *Enal-lagma* data set. Each symbol represents an individual, and the different symbols identify different populations (see legend).

the size of a dorsal tubercle. A previous genetic survey also identified differentiation among these same eastern and western populations based on amplified fragment length polymorphisms (AFLPs) (Turgeon et al. 2005). These genetic and morphological differences suggest that *E. annexum* may encompass separate eastern and western species (see also Garrison 1984). The morphology of the western *E. annexum* is very similar to that of the eastern *E. vernale*, which is found from the Atlantic coast to Michigan (Stoks et al. 2005). Although *E. vernale*'s range is completely embedded in *E. annexum*'s range (Donnelly 2004), *E. vernale* is locally allopatric from *E. annexum* in the east because *E. annexum* only inhabits ponds and lakes where dragonflies are the top predators, and *E. vernale* is found only in ponds and lakes with fish (Stoks et al. 2003, 2005). Clearly, these three species deserve substantially more basic taxonomic study.

Previous morphological studies have shown that the shapes of both male cerci and female plates have evolved over the history of the genus *Enallagma* in a fashion consistent with a model of punctuated change associated with speciation events (McPeek et al. 2008, 2009; Shen et al. 2009). Moreover, shape change in the male cerci and female plates are evolutionarily correlated (McPeek et al. 2009). This pattern of character evolution is expected for traits that drive the speciation process via changes in the species recognition system. The lack of interpopulation variation across the ranges of *Enallagma* is also consistent with cerci shape being an integral component of the species recognition system. Ferreira and Ferguson (2002) also found that song characteristics of the field cricket *Gryllus bimaculatus* used in species recogni-

tion of mates did not vary among populations. Because females acknowledge specific cues as species markers, males with more extreme cercal morphologies should have lower mating success, which would impose strong stabilizing selection on male cerci shape. The form of this stabilizing selection on male shape should not vary among populations. This system of species recognition may also impose stabilizing selection on female plate morphology as well, because females with more extreme phenotypes may also have difficulty finding acceptable mates, and if males also exercise some level of choice, more extreme females may also be rejected as possible mates.

Unlike shape, cerci size overlapped greatly among species and varied significantly among populations within most species. Because cerci size is correlated with overall body size, this interpopulation variation is not surprising because many different ecological factors influence body size (e.g., length of the growing season, resource availability), and these factors differ among lakes where *Enallagma* species are found (Siepielski et al. 2010, and in review). Body size may be under sexual selection, and variation in the form of sexual selection on body size has been demonstrated in the Eurasian damselfly *Ischnura elegans* (*Ischnura* is the sister genus to *Enallagma*) (Gosden and Svensson 2008). In fact, we have preliminary evidence that *Enallagma* male body size may experience stabilizing or directional selection depending on the relative sizes of males and females in a population at any given time (D. Steele and M. A. McPeek, unpublished data). Males that are too small relative to the size of a female may have difficulty grasping the female's thorax with his cerci, while males that are too large may not be able to position their cerci correctly on the thoracic plates of females. Thus, size could also influence species recognition, because the size of the cercus will influence the positioning of the various cerci features on the female plates: for example, even if two males had cerci of identical shape, a particular female would feel very different tactile cues if the males were quite different in size. However, male and female size positively covary across populations (M. A. McPeek, unpublished data), which will tend to maintain a match in size among males and females.

The literature is quite mixed about the importance of various evolutionary mechanisms that may influence the evolution of reproductive structures. For example, the lock-and-key hypothesis of genital fit (Dufour 1844) has long been invoked for arthropods as a means to generate species recognition and thus reproductive isolation among closely related species. However, the hypothesis has received little support in a number of empirical tests (e.g., Eberhard 2005; Mutanen et al. 2006, and see Shapiro and Porter 1989 for a general review). Likewise, intraspecific mechanisms of sexual selection and conflict are mixed in their abilities to predict patterns of intraspecific and interspecific variation in reproductive structures. Fisherian runaway sexual selection should

produce positive allometric scaling among sexual parts, but a wide diversity of scaling patterns are evident among sexual structures (Hosken and Stockley 2003), and more complicated relationships can also be derived from these mechanisms (Bonduriansky 2007; Bertin and Fairbairn 2005, 2007). Sexually antagonistic coevolution of male and female structures appear to have also shaped patterns of intraspecific and interspecific variation in some taxa (e.g., Arnqvist and Rowe 2002a,b), but not others (e.g., Eberhard 2004, 2005).

This heterogeneity in results suggests that many different evolutionary mechanisms act on reproductive structures, and the relative importance of these various mechanisms differs among taxa (Pfennig 1998). Other odonate genera (e.g., *Calopteryx*, *Hetaerina*, and *Plathemis*) have wing markings that are involved in both species recognition and various types of intraspecific sexual selection (e.g., Koenig and Albano 1987; Koenig 1991; Grether 1996; Svensson et al. 2006). For example, *Calopteryx* damselflies in both North America and Eurasia show character displacement of wing marking patterns in areas where multiple species occur sympatrically, which suggests that aspects of these marking patterns are used to discriminate conspecifics from heterospecifics (Waage 1975, 1979; Tynkkynen et al. 2004; Svensson et al. 2007). In addition, aspects of these wing markings are under sexual and countervailing natural selection pressures, which can cause populations within species to diverge in aspects of these marking patterns as well (e.g., Svensson et al. 2004). Likewise, other taxa also have aspects of their phenotypes that are involved in discriminating conspecifics from heterospecifics and choosing among conspecifics: for example, the whine and chuck components of the túngara frog's (*Physalaemus pustulosus*) call (Ryan et al. 2003; Phelps et al. 2006); olfactory cues and body striping versus body size in the pygmy swordtail (*Xiphophorus pygmaeus*) (Hankison and Morris 2002, 2003).

While species recognition cues must be different among species to be effective, the preference of the choosing sex must also evolve to generate an effective species recognition system (Pfennig 1998; McPeek and Gavrilets 2006). Consistent with this, *Calopteryx* males that are sympatric with another *Calopteryx* species are more discriminating of heterospecifics than males from areas where they are the only *Calopteryx* species (Waage 1975; Wellenreuther et al. 2010). The corollary prediction from this is that the levels of population variation in species recognition cues and preferences will be greater for species that occur with only a few but different species across their range, and the variance within populations may be inversely related to the number of sympatric species (McPeek and Gavrilets 2006; Hoskin and Higgie 2010). This may in fact be why *Enallagma* show such low levels of variation in cerci shape across species ranges. Over much of North America 3–12 *Enallagma* species can be found at almost every permanent pond and lake (e.g., Johnson and Crowley

1980; McPeek 1998; Butler and deMaynadier 2008), and because most species have extensive ranges, each species must interact with largely the same collection of other species over broad geographic areas (Donnelly 2004).

The results of this study indicate that traits signaling species identity were nonoverlapping among species, and varied little between populations within species. If these are the traits that define species boundaries, the next logical question is how do differences in these traits arise if they experience such strong and uniform stabilizing selection within species? All the species in this study are very closely related and differ very little at both the genetic and ecological levels (McPeek 1998; Turgeon et al. 2005), which suggests that changes in these signals may have been the primary driver of speciation. Whether changes in these traits are the primary cause of speciation or an ancillary change that accompanies speciation by other means is the next critical question to address.

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