# MORPHOMETRIC INFERENCES ON SIBLING SPECIES AND SEXUAL DIMORPHISM IN *NEOCHLAMISUS BEBBIANAE* LEAF BEETLES: MULTIVARIATE APPLICATIONS OF THE THIN-PLATE SPLINE

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*Abstract.*—Nominally polyphagous species of herbivorous insects sometimes are comprised of multiple morphologically similar biological species with more specialized appetites. When meristic morphological traits cannot be found to distinguish such suspected sibling species, molecular data are increasingly sought as a source of evidence. A role for morphology in distinguishing such taxa might be reclaimed, however, by recent advances in geometric morphometric methods, such as the statistical analysis of partial-warp scores from the thin-plate spline. We employed this method to detect and characterize subtle shape differences among populations and between sexes of the nominal leaf beetle species *Neochlamisus bebbianae*. Using the thin-plate spline, the shapes of specimens from seven beetle populations collected from five host plants in five eastern North American localities were calculated. These shapes were analyzed by MANOVA, revealing significant variation in both uniform and nonuniform components of shape among test populations. Significant sexual dimorphism in size, shape, and allometric relationships were also documented across these populations. More interestingly, our study provided evidence of sibling species where traditional taxonomic approaches have failed. Individual MANOVAs revealed significant shape variation between sympatric populations from different host plants in each of three localities. Because these sympatric shape differences were significant when adjusted for size, they cannot be attributed to allometric consequences of size variation among test populations. Because certain beetle populations differed significantly in size and shape when reared in a common environment, these morphometric traits may have a genetic basis. Together, these results are consistent with an earlier suggestion that *N. bebbianae* represents a complex of host-specific races or sibling species, a hypothesis that has received additional support from recent studies on host use traits, sex ratios, and mitochondrial DNA. In sum, these analyses demonstrate the power and utility of the thin-plate spline as a morphological means of discriminating among closely related and anatomically homogeneous taxa. [Chrysomelidae; host races; morphometrics; *Neochlamisus*; relative warps; sexual dimorphism; sibling species; thin-plate spline.]

When separate biological species are extremely similar in morphology, documenting their existence is often difficult and many complexes of such cryptic or sibling species go undetected, languishing under single nominal species names (Mayr, 1942). This is especially true when museum specimens and traditional taxonomic approaches provide the only available evidence on biological variation. Observations on behavioral or ecological polymorphisms, however, may hint at such sibling species and have proven especially informative in systematic studies of herbivorous insect taxa. In 1864, Benjamin Walsh described slight but consistent morphological differences between nominally conspecific populations of insects associated with different host plants at single localities. From the strict segregation of these characters, he inferred that interbreeding did not occur between such sympatric populations, which he accordingly diagnosed as separate "phytophagic species." Walsh's approach has since been used to ferret out many host-specific biological species from within single nominal species assumed to have more generalized feeding habits (Brown, 1958, 1959; Diehl and Bush, 1984).

The recognition of cryptic biological species is of taxonomic significance and is also vitally important for evolutionary biologists, ecologists, and applied biologists who often assume the evolutionary and genetic coherence of the nominal species that they study. Data on "intraspecific" differentiation in host use, patterns of ecological specialization, or the efficacy of biological control agents, for example, may demand quite different interpretations if

nominally conspecific study populations actually belong to separate, host-specific sibling species. However, although morphological data are commonly a valuable source of phylogenetically informative characters (Hillis, 1987; Patterson et al., 1993), even careful taxonomic scrutiny may fail to furnish morphological apomorphies that discriminate among extremely similar taxa from different hosts. Fortunately, even morphologically identical species can sometimes be distinguished using molecular data such as allozymes or DNA sequences. Recently, molecular genetic approaches have documented evolutionary differentiation among host-associated populations within a number of nominal herbivore species (e.g., Guttman et al., 1981; Diehl and Bush, 1984; Berlocher, 1989; Menken, 1989; Waring et al., 1990; Roininen et al., 1993; Brown et al., 1996; Funk, 1996). Must morphology, then, wholly cede its diagnostic role to the molecules when closely related species have not diverged in meristic morphological traits? In such instances, morphology might still play a role through the quantitative analysis of continuously varying shape characters, the domain of morphometrics.

In traditional morphometric analyses, linear distances are measured between pairs of apparently homologous morphological points, known as landmarks (Blackith and Reyment, 1971; Marcus, 1990, 1993). These interlandmark distances are then analyzed using multivariate statistics. One limitation of the traditional approach, however, is its assumption that these distances, too, are homologous across taxa, even though each distance reflects the (possibly independent) evolution of the structures that it spans (Bookstein, 1982, 1990). A second limitation is the partial redundancy of information about shape provided by any two distances, yielding weaker statistical tests of morphological differences (Strauss and Bookstein, 1982). Because of these shortcomings, morphometricians are now describing two- and three-dimensional forms by capturing and analyzing the Cartesian coordinates of homologous landmarks (Bookstein, 1989,

1991; Rohlf and Marcus, 1993). Such geometric morphometric methods allow shape to be described more comprehensively by preserving the positional relationships among landmarks, thus minimizing redundancy and allowing for more powerful statistical tests of shape differences. In this paper, we characterize shape variation using a recent advance in geometric morphometric methodology: the analysis of partial-warp scores using the thin-plate spline (Bookstein, 1989, 1991). These analyses were performed using data from host-associated populations of the nominal leaf beetle species, *Neochlamisus bebbianae* (Brown).

*Neochlamisus* (Karren, 1972) is a genus of North American chrysomelid beetles that feed on the foliage of their host plants as both larvae and adults. Although members of this genus as a whole use host plants from a taxonomically disparate array of plant families (Karren, 1972), most species appear to have monophagous tendencies, primarily using a single host genus or even a single species. The nominal species *N. bebbianae*, however, appears to be more generalized in its feeding habits. Field collections and laboratory studies document its use of six plant genera from four divergent families of angiosperms (Brown, 1943, 1946, 1952; Funk, 1996, unpubl. data): *Acer rubrum* (red maple, Aceraceae), *Alnus rugosa / A. serrulata* (speckled alder/hazel alder, Betulaceae), *Betula nigra* (river birch, Betulaceae), *Corylus americana* (American hazel, Betulaceae), *Quercus* spp. (oak, Fagaceae), and *Salix bebbiana* (Bebb's willow, Salicaceae).

In his studies of these beetles in Ontario, Canada, Brown (1943, 1946, 1952) observed that local populations sometimes used one of these hosts and ignored others. He also noted subtle color differences among series of beetles associated with different host plants. These observations convinced Brown that all species of *Neochlamisus* were highly specialized and that each *N. bebbianae* host thus supported a separate, host-specific sibling species. Having studied specimens from four hosts, *Alnus, Corylus, Salix,* and *Quercus,* Brown de-

scribed populations associated with the first three as separate sibling species and suggested that *Quercus* populations likely represented a fourth sibling (Brown, 1943, 1946, 1952).

However, in his revision of *Neochlamisus*, Karren (1972) found no consistent morphological differences among specimens from these populations in relation to their host of origin despite his examination of internal male genitalia, which often identify externally indistinguishable insect species (Eberhard, 1985). Karren thus synonymized Brown's sibling species under *N. bebbianae*, concluding that the host-associated forms were simply populations of a single, more ecologically generalized species. Karren also referred particular specimens collected from *Acer* and *Betula* (host associations unknown to Brown but since confirmed by D.J.F.) to *N. bebbianae*. Based on the study of larval characters, LeSage (1984) resurrected the idea that *Alnus*- and *Salix*-using forms belong to separate sibling species. Clearly, additional sources of data are needed to address the evolutionary status of host-associated populations presently assigned to the nominal species *N. bebbianae*.

Another intriguing aspect of *Neochlamisus* biology is the unusual patterns of sex allocation exhibited by various species, including *N. bebbianae*. Population sex ratios are often strongly female biased (unpubl. data), and many species are apparently sexually dimorphic in size, with females larger than males (Karren, 1972). Qualitative observations on interspecific patterns of sexual size and shape dimorphism (D.J.F., pers. obs.) seem to parallel those revealed by recent quantitative investigations of sexual dimorphism and its evolutionary causes in water striders (e.g., Fairbairn, 1990, 1992; Fairbairn and Preziosi, 1994). These patterns suggest a further avenue of evolutionary study that might be advanced by morphometric approaches.

In this study, we employed a recent advance in geometric morphometric methodology to identify and describe shape variation between sexes and among host-associated populations of *Neochlamisus beb-*

TABLE 1. Data on populations of *Neochlamisus bebbianae* collected from five of its six well-documented host plants. * = plants believed by Brown (1943, 1946, 1952) to support host-specific sibling species within *N. bebbianae*. No beetles were collected for study from the host of the fourth purported sibling, hazel. The Quebec *Alnus*-associated population was not treated in the original analyses.

| Location | Host plant | Beetle specimens | |
|----------|-----------|------|---|
| | | Sex | *n* |
| Georgia | *Acer rubrum* | female | 30 |
| Georgia | *Acer rubrum* | male | 30 |
| Georgia | *Betula nigra* | female | 30 |
| New York | *Acer rubrum* | female | 30 |
| New York | *Acer rubrum* | male | 30 |
| Oklahoma | *Quercus* spp.* | female | 30 |
| Oklahoma | *Betula nigra* | female | 30 |
| Oklahoma | *Betula nigra* | male | 30 |
| Ontario | *Salix bebbiana** | female | 30 |
| Ontario | *Salix bebbiana** | male | 30 |
| Ontario | *Alnus incana** | female | 30 |
| Quebec | *Alnus incana** | female | 30 |

*bianae*. Our goals were (1) to provide a further morphological test of the hypothesis that *N. bebbianae* includes host-specific sibling species, (2) to assay patterns of sexual dimorphism that might point towards further research, and (3) to reclaim for morphology a role increasingly played by molecular genetic analyses, that of discerning differences between closely related and morphologically similar populations and taxa.

## MATERIALS AND METHODS

### Collection of Specimens

The specimens treated in this study were collected from several regions spanning eastern North America: northeastern Georgia; Suffolk County, New York; Latimer County, Oklahoma; Ottawa, Ontario; and Cowansville, Quebec (Table 1). These specimens were collected from five of the six well-documented host plants of *N. bebbianae*, leaving *Corylus* as the only host not represented by a test population in this study. Animals from each Georgia, Oklahoma, and New York study population were collected from a number of sites within each region, but those from Ontario and Quebec were each collected from a single site. The specific specimens from

each of the host-associated populations (Table 1) that were used for analysis were randomly selected from beetles pooled across collecting sites within a given region.

In three cases, study specimens were collected from two different hosts in a single region, allowing comparisons of sympatric (or quasi-sympatric) host-associated populations. In Ontario, *Alnus*- and *Salix*-associated beetles were collected from plants that intermingled in the same fields. In Georgia, some *Acer* and *Betula* beetles were collected from the same sites, where their hosts intermingled along streams. In Oklahoma, although both *Quercus* and *Betula* beetles were not collected from intermingling host plants at any single collection site, distances between collection sites for the two hosts were as little as a few hundred meters, whereas distances between collection sites from a single host were as great as 20 km. Thus, collection sites for the two hosts were themselves spatially intermingled at a larger geographic scale.

From each test population, 30 animals from a given sex were collected for analysis. Owing to the rarity of males, only females were examined for some populations (Table 1). All test animals were collected directly from their host plant, in most cases as eggs or young larvae, which were subsequently reared to eclosion on foliage from the host on which they were collected. Ten of the 30 specimens of Oklahoma *Betula* males and females and 10 of the 30 Oklahoma *Quercus* females were collected as adult beetles, however, and some of the *Alnus* and *Salix* beetles from Ontario and Quebec were collected as late instar larvae or pupae.

### Data Collection

Eleven landmarks were chosen for their capacity to define major elements of shape and for their reliability as homologous biological structures (Fig. 1; Table 2). Based on Bookstein's (1991) classification, five of these landmarks are of type I, representing meeting points of two or more tissues, and six are of type II, marking points of max-
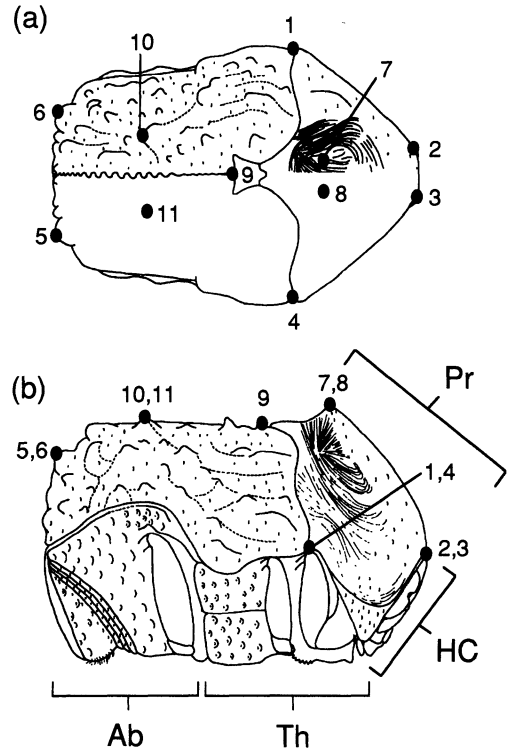


FIGURE 1. Dorsal (a) and lateral (b) views of the 11 landmarks used to define the shape of *Neochlamisus bebbianae* specimens (see description of landmarks in Table 2). For each view, the anterior of the animal is to the right. HC = head capsule; Th = thorax; Pr = pronotum; Ab = abdomen. Illustrations reproduced from Karren (1972).

imum curvature. Two-dimensional coordinates for these landmarks were collected from all 330 specimens using a Wild dissecting microscope, a CCD video camera, and the program MorphoSys (Meacham and Duncan, 1990). Specimens were scored in random order with respect to population of origin.

### Choosing a Geometric Morphometric Method

A variety of geometric morphometric methods have been developed to analyze two-dimensional landmark data such as those collected from our study specimens. With Euclidean distance matrix analysis (Lele and Richtsmeier, 1991; Richtsmeier et al., 1992), the shapes of two specimens are compared by calculating ratios of their in-

TABLE 2. Morphological landmarks used in this study, as classified by Bookstein (1991). See Karren (1966) for definitions of morphological terms.

| Landmark | Description | Type |
|---|---|---|
| 1 | junction of pronotum and left elytron | I |
| 2 | junction of pronotum and left margin of head capsule | I |
| 3 | junction of pronotum and right margin of head capsule | I |
| 4 | junction of pronotum and right elytron | I |
| 5 | apex of most posterolateral tubercle (su-3a) on right elytron | II |
| 6 | apex of most posterolateral tubercle (su-3a) on left elytron | II |
| 7 | apex of left pronotal gibbosity (=hump) | II |
| 8 | apex of right pronotal gibbosity (=hump) | II |
| 9 | junction of mesoscutellum, right elytron, and left elytron | I |
| 10 | apex of su-2 tubercle on left elytron | II |
| 11 | apex of su-2 tubercle on right elytron | II |

terlandmark distances for all possible distances (Lele and Richtsmeier, 1991). Shape differences increase as these ratios depart from unity. Although this method allows for the statistical description of shape, only two specimens can be compared at a time and visualization of shape variation is not possible (see Rohlf et al., 1996). Finite-element scaling analysis (Cheverud et al., 1983) adopts an engineering perspective, decomposing the form of each specimen into a composite of small geometric elements and calculating the "strain" necessary to deform the elements of one specimen into those of the other (Cheverud and Richtsmeier, 1986). Unfortunately, results from this method are sensitive to the (arbitrary) choice of the elements employed (Bookstein, 1991). The analysis of superimposed specimens (e.g., Walker, 1993) describes shape variation by comparing individual specimens with a consensus configuration, representing the average Cartesian coordinates for each landmark across all specimens (Bookstein, 1991; Rohlf, 1993b). In this approach, specimens are first superimposed on one another so

that the deviations of landmarks are minimized based on some criterion (Rohlf and Slice, 1990). The residuals from this superimposition are then treated as shape data and used directly for group comparisons (Walker, 1996). Both median-based (Siegel and Benson, 1982) and least squares or Procrustes (Sneath, 1967; Gower, 1971) methods have been proposed. Although the Procrustes method is the most mathematically convenient criterion, empirical studies have indicated that both methods generate nearly identical results when sample sizes and the number of landmarks are large (Slice, 1993). Although the analysis of superimposed specimens allows for a description of total shape variation, it does not allow for the partitioning of shape into uniform and nonuniform components. Such partitioning of shape variation is only possible with the thin-plate spline.

Using the thin-plate spline (Bookstein, 1989, 1991), variation among specimens is expressed as variation in the parameters of an interpolation function that compares a given specimen to the consensus configuration. These parameters are treated as descriptors of shape, much as the parameters from a Fourier analysis are used to describe the outline of a curve (see Rohlf, 1990). Specimens are first superimposed using the Procrustes method because this distance measure is assumed by Kendall's (1984) shape space, which is used by the thin-plate spline (Bookstein, 1996a). A consensus configuration is determined, and the thin-plate spline function is calculated. This function is then decomposed into a number of geometrically orthogonal elements called principal warps. These warps are a set of multidimensional shape axes describing a set of deformations of various magnitudes that together account for the entire shape of the consensus configuration. Superimposed specimens are projected onto these principal warps to describe their deviations from the consensus configuration (Bookstein, 1989; Rohlf, 1993b). These projections, or partial-warp scores, are used as data in multivariate analyses (Rohlf, 1993b; Rohlf et al., 1996).

Shape deformations can also be mathematically partitioned into uniform and nonuniform components. Uniform components represent simple overall linear stretching or compression of specimens, whereas nonuniform components characterize more complex changes in shape, such as nonlinear patterns of deformation where changes can be localized to small regions on specimens. The thin-plate spline is particularly appealing because it allows the graphical depiction of shape variation as deformations in a manner similar to D'Arcy Thompson's (1917) transformation grids, where dramatic and subtle variations alike can be appreciated visually. For these reasons, we have adopted the thin-plate spline to analyze shape variation among populations of *N. bebbianae*.

### Morphometric and Statistical Analyses

Centroid size, the square root of the sum of the squared distances from each landmark to the centroid (i.e., the center of gravity), was calculated for each specimen, and Procrustes superimposition of specimens was provided by the computer program GRF-ND (Slice, 1994), allowing the consensus configuration to be determined. Bookstein's (1993, 1996b) uniform shape components and nonuniform shape variables (partial-warp scores) were then calculated using the thin-plate spline program in NTSYS-pc (TPSWTS; Rohlf, 1993a), generating a set of 16 partial-warp scores for each specimen. To identify and describe major trends in nonlinear shape variation, we performed a principal components analysis on the matrix of partial-warp scores (the $W$ matrix) (Rohlf, 1993b). In such an analysis, the first principal component axis, i.e., the first relative warp (Bookstein, 1991), represents the direction of maximal nonuniform shape variation among all specimens (Rohlf, 1993b). When calculating the relative warps, we set the scaling parameter, $\alpha$, to 0, as recommended by Rohlf (1993b) for taxonomic studies. A value of 0 weights all partial-warp scores equally so that neither large-scale nor small-scale shape differences are emphasized. Although different values of $\alpha$

affect the description of shape variation by relative warps, they do not affect statistical inferences based on a MANOVA and canonical variates analysis because these statistical methods are scale free.

To determine if shape varied significantly among test populations and between the sexes, we performed a two-way MANOVA on both the uniform ($u1$ and $u2$) and nonuniform ($W$ matrix) components of shape. Because no evidence of interactions between test population and sex were found, we were able to perform a canonical variates analysis to identify the patterns of shape similarity among populations. Following these analyses, we specifically compared pairs of populations from both the Georgia and Oklahoma sites using a MANOVA to determine whether sympatric populations from different hosts differed in shape. We also compared the allometric slopes of sympatric populations using a MANCOVA to determine whether shape differences among populations were simply the allometric consequence of size differences among populations. Both analyses were performed using females only because one of the two populations in each comparison was not represented by male specimens. Following the completion of the original analyses, *Alnus*-associated specimens were obtained from Ontario, allowing a third sympatric comparison (with Ontario *Salix* beetles). The MANOVA and MANCOVA were likewise applied to these populations. Although the sympatric comparisons had been conceived a priori, the remaining 19 population comparisons were made a posteriori after transforming the generalized distances from the canonical variates analysis into Hotelling $T^2$ values (Rohlf, 1993a). Using the Bonferroni method (Sokal and Rohlf, 1995), an experiment-wise error rate of 0.05 was maintained by dividing by the number of unplanned comparisons (19), to obtain a critical $\alpha$ of 0.0026.

We investigated sexual dimorphism by (1) comparing male and female centroid size to determine if the sexes were dimorphic in size, (2) determining from the overall MANCOVA if the sexes were dimorphic
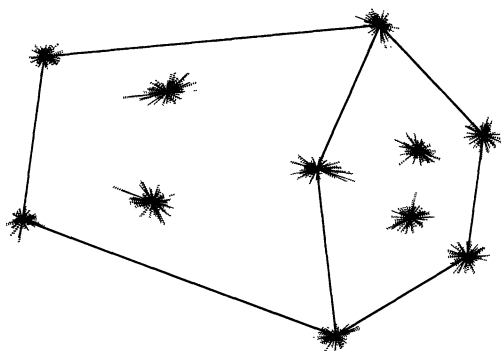
FIGURE 2. Generalized least squares (Procrustes) superimposition of 330 specimens of *N. bebbianae* showing the range of variation at each landmark after translation, rotation, and scaling of data from individual specimens. The outline formed by connecting the mean coordinates of each landmark represents the consensus configuration for an "average" *N. bebbianae* specimen.

in shape, (3) performing a multivariate regression of shape on size to determine if overall shape variation had an allometric component, and (4) performing a MANCOVA with sex as the categorical variable and centroid size as the covariate to determine if sexual dimorphism in shape was an allometric consequence of dimorphism in size. We also generated representations of male and female shapes by regressing the partial-warp scores onto a dummy variable with a separate state for each sex. All statistical analyses were performed using NTSYS-pc (Rohlf, 1993a) and SAS.

## RESULTS

### General Description of Shape Variation

Figure 2 depicts variation among test specimens at each landmark and illustrates an average *N. bebbianae* study specimen by connecting the mean values for each landmark, which together define the consensus configuration. The first relative warp (RW1) explained 29% of shape variation among specimens, and the first three relative warps combined to explain 58% of this variation. Shape variation along RW1 illustrates sources of deformation from the consensus configuration (Fig. 3). At the positive extreme of RW1, internal land-

(a) **+ deviation**
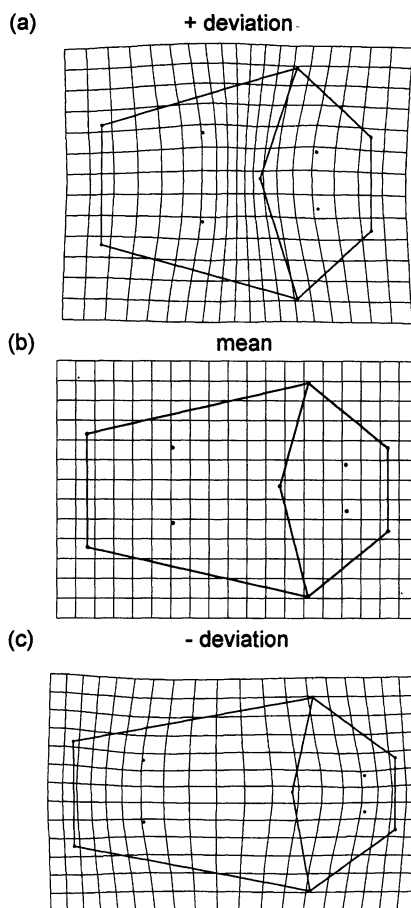
(b) **mean**

(c) **- deviation**



FIGURE 3. Overall deformations in shape along the first relative warp for all 330 beetle specimens. This figure represents the direction of maximal nonuniform shape variation obtained from a principal components analysis of the partial-warp scores (W matrix). The range of observed shape variation along this axis is presented. The broader, squatter shapes illustrate positive deformations (a) and the narrower, more elongate shapes show negative deformations (c) from the consensus configuration (b).

marks (7–11 in Fig. 1) are closely arranged and outline landmarks (1–6) define a relatively squat shape (Fig. 3a) in which the pronotum (defined by landmarks 1–4 and 9) occupies a large proportion of the total surface area. At the negative extreme of RW1, internal landmarks are more highly dispersed, and outline landmarks define a more elongate shape (Fig. 3b) with a relatively smaller pronotum.

TABLE 3. Two-way MANOVA for uniform and nonuniform (**W** matrix) components of shape. Analyses treat all 330 beetle specimens, using two variables for uniform components and $2p - 6$ (=16) variables for nonuniform components.

| Source | df | Wilks $\Lambda$ | parms | F | df | P |
|---|---|---|---|---|---|---|
| Uniform | | | | | | |
|   Host plant | 4 | 0.9022 | 2, 4, 320 | 4.24 | 8, 642 | 0.0001*** |
|   Sex | 1 | 0.9891 | 2, 1, 320 | 1.77 | 2, 321 | 0.1720 |
|   Host × Sex | 4 | 0.9962 | 2, 4, 320 | 0.31 | 4, 642 | 0.8727 |
| Nonuniform | | | | | | |
|   Host plant | 4 | 0.6068 | 16, 4, 320 | 2.56 | 64, 1204 | 0.0001*** |
|   Sex | 1 | 0.8689 | 16, 1, 320 | 2.89 | 16, 307 | 0.0002*** |
|   Host × Sex | 4 | 0.9116 | 16, 4, 320 | 0.91 | 32, 614 | 0.6136 |

## Shape Differentiation among Test Populations

Two-way MANOVAs on both uniform and nonuniform components of shape revealed significant variation among test populations (Table 3), as did canonical variates analysis (Wilks $\Lambda = 0.47884$, $P = 2 \times 10^{-13}$). A minimal spanning tree based on generalized distances illustrated the relative similarity of populations separated in shape space by ordination along the first two canonical axes (Fig. 4; Sneath and Sokal, 1973). Projecting specimens onto the canonical vectors and performing a regression of partial-warp scores on each canonical axis illustrated implied deformations in shape along these axes (Fig. 4).

For all three regions in which beetles were collected from two host plants, highly significant shape differences were detected between these sympatric, host-associated populations (Georgia: Wilks $\Lambda = 0.6154$, $P = 0.0012$; Oklahoma: Wilks $\Lambda = 0.5788$, $P = 0.0002$; Ontario: Wilks $\Lambda = 0.1431$, $P = 0.0001$). Additionally, 6 of the 19 possible unplanned comparisons among allopatric populations showed significant shape differentiation, even at a critical $\alpha$ of 0.0026: Georgia *Acer* versus Quebec *Alnus*, Ontario *Salix*, and Oklahoma *Quercus*; Oklahoma *Betula* versus Georgia *Betula* and Ontario *Salix*; and Ontario *Salix* versus Oklahoma *Quercus* (Table 4).

For the sympatric Georgia and Oklahoma comparisons, no differences were found between the allometric slopes of the sympatric populations (Georgia: Wilks $\Lambda = 0.7065$, $P = 0.4167$; Oklahoma: Wilks $\Lambda = 0.8508$, $P = 0.8512$). However, in each case, population shapes were significantly different when size was held constant allometrically (Georgia: Wilks $\Lambda = 0.5346$, $P = 0.0164$; Oklahoma: Wilks $\Lambda = 0.5464$, $P = 0.0222$). These populations thus have different intercepts, so population shape differences are not simply allometric projections of population size differences. The sympatric Ontario comparison did show significantly different allometric slopes between populations (Wilks $\Lambda = 0.5345$, $P = 0.0200$), again revealing separate allometric trajectories.
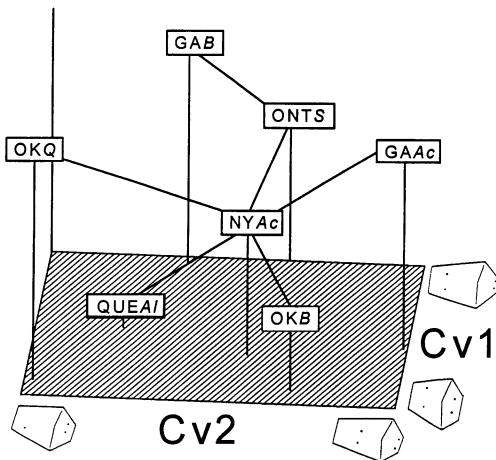


FIGURE 4. Canonical variates ordination of beetle test populations, presented as a minimal spanning tree. Beetle shapes are thin-plate spline representations of the deformations implied by variation along the first and second canonical axes. State or province abbreviations for collection localities are followed by abbreviations for host plant: *Ac* = *Acer*; *Al* = *Alnus*; *B* = *Betula*; *Q* = *Quercus*; *S* = *Salix*.

TABLE 4. Multiple comparisons of nonuniform shape components among test populations. † = significant planned comparisons using MANOVA; * = significant unplanned comparisons based on Hotelling's $T^2$ values with Bonferroni adjustment for critical $\alpha$. The Ontario *Alnus* test population is not treated in these comparisons because its specimens were collected after these analyses were completed.

| | GA *Acer* | GA *Betula* | NY *Acer* | OK *Quercus* | OK *Betula* | ONT *Salix* | QUE *Alnus* |
|---|---|---|---|---|---|---|---|
| GA *Acer* | 0 | | | | | | |
| GA *Betula* | 39.8† | 0 | | | | | |
| NY *Acer* | 23.8 | 30.8 | 0 | | | | |
| OK *Quercus* | 64.8* | 44.9 | 33.8 | 0 | | | |
| OK *Betula* | 39.8 | 57.8* | 15.0 | 48.7† | 0 | | |
| ONT *Salix* | 53.1* | 28.8 | 38.3 | 71.4* | 60.5* | 0 | |
| QUE *Alnus* | 59.2* | 31.5 | 29.8 | 55.3 | 45.0 | 44.4 | 0 |

### Shape Variation and Sexual Dimorphism

Comparison of centroid sizes showed that females were significantly larger than males ($F = 17.09$, $P = 5.2 \times 10^{-15}$), supporting Karren's (1972) observations. How-
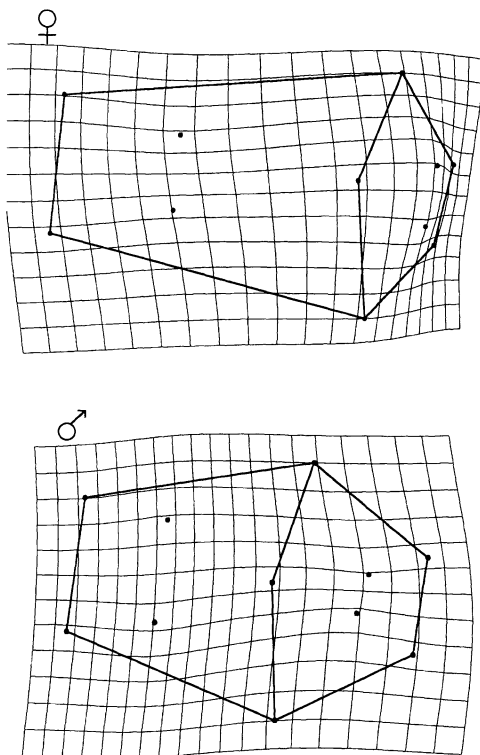


FIGURE 5. Predicted female and male shapes represented as thin-plate splines. Females are more elongate, with more anterior pronotal gibbosities and pronotum/elytrum junctions. Males are squatter, with the pronotal gibbosities and pronotum/elytrum junctions positioned more posteriorly.

ever, the two-way MANOVA revealed that the sexes differed significantly in nonuniform (although not in uniform) components of shape (Table 3). Predicted males are squatter with more posteriorly positioned pronotal gibbosities and pronotal/elytral junctions, and females are more elongate with more anteriorly positioned gibbosities and junctions (Fig. 5). Females also appear to allocate a much larger proportion of the body to the posterior segments of the thorax and the abdomen than do males.

A statistically significant relationship between shape and size across all test specimens ($R^2 = 0.2971$, $F = 8.27$, $P < 0.0001$) illustrated significant allometry. Although allometric slopes did not differ significantly between the sexes (Wilks $\Lambda = 0.9695$, $P = 0.8720$), shape was significantly sexually dimorphic when size was held constant allometrically, meaning that the intercept for males and females differed. This suggests that males and females vary along separate, although parallel, allometric trajectories.

### DISCUSSION

#### Morphometric Evidence of Sibling Species

Our multivariate analyses of partial-warp scores suggest that certain host-associated populations currently assigned to the nominal leaf beetle species *Neochlamisus bebbianae* may represent separate evolutionarily differentiated taxa. In each of three cases where sympatric test populations were collected from separate host plants, shape differences between these

populations were highly significant. Such local shape differentiation might be explained by reproductive isolating mechanisms that restrict the homogenizing effects of gene flow between sympatric populations. This study thus demonstrates the capacity of thin-plate spline analysis to detect subtle morphological evidence of biological species that may have eluded the taxonomist relying on meristic characters (Karren, 1972). More specifically, shape differentiation between *Alnus*- and *Salix*-associated populations in Ontario supports the contention of Brown (1943) and LeSage (1984) that these host forms belong to separate sibling species, differentiation between *Quercus* and *Betula* beetles in Oklahoma is consistent with Brown's (1952) suspicion of a *Quercus*-specific sibling, and differentiation between *Betula* and *Acer* beetles in Georgia suggests that these hosts, unexamined by Brown, may also support separate sibling species.

Before sympatric shape differences are interpreted as evidence for reproductive isolation and genetic differentiation, however, the possibility that they are environmentally induced must be considered. The host-associated populations currently assigned to *N. bebbianae* might belong to a single biological species with generalized feeding habits that develops different adult shapes depending on its larval host. Such trophically induced polymorphisms have been documented, for example, in cichlid fishes (Meyer, 1987). Host plant-induced shape variation, however, has rarely been found in herbivorous insects; one exception is Gillham and Claridge's (1994) study of *Alnetoidia alneti* leafhoppers. Adult leafhoppers collected from two host plant species as adults differed significantly in shape, but those collected from these two host plants as young nymphs and then reared to maturity on a single host species did not exhibit different shapes.

Such direct induction of adult shape by juvenile feeding experience is, however, more plausible in hemimetabolous leafhoppers than in holometabolous beetles. Whereas hatchling leafhoppers are essentially miniature adults that grow and sprout wings during development, *Neochlamisus* larvae are grublike creatures that metamorphose into adults with a strikingly different appearance. During metamorphosis, larval tissues are almost entirely broken down before adult structures are built anew. At this time, any host plant-induced changes in larval shape are likely to be lost, just as host plant-induced changes in feeding preferences generally are (Jaenike, 1990:249). Because adults do not grow and because we selected landmarks from rigid elytral and pronotal structures, it is also unlikely that observed shape differences were influenced by adult feeding experience. Our rearing of *Betula*-associated larvae from Georgia and Oklahoma on the same host material under identical laboratory conditions provided a post facto common garden experiment showing that *N. bebbianae* populations may exhibit significant shape variation even when larval environment is held constant (Table 4). These results suggest that shape differences may have a genetic basis.

Although herbivore shape may rarely be directly induced by larval host plant, herbivore size may vary according to the suitability of the host for larval development. If beetle shape varies as an allometric consequence of such size variation, larval host association might thus indirectly induce population shape differences. Such an allometric component of shape variation was documented within *N. bebbianae* test populations. However, because allometric trajectories differed significantly between sympatric populations in every case, the shape differences between these populations cannot be attributed to differences in size. Furthermore, our post facto common garden experiments also suggest that size variation among populations may itself be genetically based rather than host induced. In both cases where two test populations were collected from the same host and reared on its foliage in a common laboratory environment, adults of the two populations differed significantly in size (Georgia and New York *Acer*: $F = 23.34$, $P < 0.0001$; Georgia and Oklahoma *Betula*: $F = 15.57$, $P < 0.0001$).

Our morphometric inferences on the existence of host-associated taxa within *N. bebbianae* receive further support from other data sources in each case: Ontario *Alnus*- and *Salix*-associated populations exhibit strikingly different sex ratios, and although each will feed on the other's host, each prefers its native host foliage in feeding trials (unpubl. data); Oklahoma *Betula*- and *Quercus*-associated populations entirely reject one another's hosts in no-choice feeding trials (unpubl. data); and *Betula*- and *Acer*-associated populations from Georgia largely reject one another's hosts across a number of host preference and performance assays (Funk, 1996). Furthermore, *Betula* beetles (and to a lesser extent *Alnus* beetles) lack the variable degree of bluish tint that characterizes *Acer* and *Salix* beetles, supporting Brown's belief in the diagnostic value of color. These results suggest the genetic differentiation of these sympatric population pairs. Insights into the history of gene flow between them are provided by other recent studies. Mating experiments revealed significant sexual isolation between allopatric *Acer*- and *Betula*-associated populations. Mitochondrial DNA (mtDNA) sequences collected from sympatric *Acer* and *Betula* beetles in Oklahoma sort monophyletically according to host plant, suggesting a lack of recent gene flow between them, consistent with the hypothesis that they represent completely reproductively isolated sibling species (Funk, 1996). However, certain Canadian *Alnus*- and *Salix*-associated beetles share mtDNA haplotypes, suggesting a more recent history of gene flow between populations that may represent incompletely isolated host races rather than completely isolated sibling species (for a discussion of terms, see Jaenike, 1981; Diehl and Bush, 1984). The limited number of comparisons in the present study are insufficient for diagnosing the actual number and host specificity of evolutionarily differentiated host forms within *N. bebbianae*. They also offer no evidence on whether the reproductive isolation between these host forms originally evolved in allopatry or sympatry. Addressing these issues will require con-siderable genetic and experimental analysis.

### Morphometric Evidence of Sexual Dimorphism

Our analyses also allowed us to characterize morphological variation between the sexes. Comparison of centroid sizes showed that *N. bebbianae* females are significantly larger than males, statistically supporting Karren's (1972) observation. Such female-biased sexual size dimorphism is commonly observed in invertebrate taxa. Using more traditional morphometric measures, three recent studies of invertebrates (Wickman and Karlsson, 1989 [butterflies]; Fairbairn, 1992 [water striders]; Prenter et al., 1995 [orb-weaving spiders]) have documented another interesting pattern: sexual dimorphism in shape attributable to relatively larger female abdomens. Such shape dimorphism could be the result of selection on increased female fecundity, a hypothesis supported by the correlation of fecundity with female abdomen size in the common water strider *Aquarius remigis* (Preziosi et al., 1996). If fecundity typically increases with female abdomen size, Wickman and Karlsson's (1989) finding that insect abdomen size increases allometrically with body size suggests that the pattern of sexual size dimorphism described above may also be due partly to fecundity selection. Our findings of female-biased size dimorphism and of sexual dimorphism in shape that might be attributable to the relatively larger abdomens of females (Fig. 5) suggest that *N. bebbianae* may offer a useful systems for studying these issues.

Studies of sexual dimorphism in taxa with larger males have commonly revealed an interspecific pattern of hyperallometry: as size increases, the degree of sexual size dimorphism increases. In a taxon with larger females (water striders), Fairbairn (1990; Fairbairn and Preziosi, 1994) documented a complementary pattern of hypoallometry: as size increases, the degree of sexual size dimorphism decreases. These patterns suggest a role for sexual selection on male size. Likewise, within *Neo-*

*chlamisus* (1) females are the larger sex, (2) the largest known species is clearly the least dimorphic in size, whereas smaller species seem to exhibit stronger sexual size dimorphism (Funk, pers. obs), (3) significantly different allometric trajectories for male and female *N. bebbianae* suggest that these interspecific patterns of shape dimorphism could have an allometric component, and (4) a role for sexual selection is hinted at by no-choice matings studies showing *N. bebbianae* copulations to be somewhat more likely, more rapidly initiated, and of longer duration on average when male : female size ratios of paired animals are greater (Funk, 1996).

### Studying Shape Differentiation with the Thin-Plate Spline

Despite its advantages, the use of the thin-plate spline in biological studies has thus far been limited. Formulations of the method by Bookstein (1989, 1991) included its application to studies of rat skull development, fossil Foraminifera, and human patients with Apert's syndrome. Rohlf (1993b) compared mosquito wings to demonstrate the merits of including a scaling paramenter, α. Other applications include studies of squirrel scapulas (Swiderski, 1993), the ontogeny of piranha (Fink and Zelditch, 1995), rat skulls (Zelditch et al., 1992), and mole skulls (Loy et al., 1993; Rohlf et al., 1996). Using a somewhat controversial approach (F. L. Bookstein and F. J. Rohlf, pers. comm.), Zelditch et al. (1995) recently applied thin-plate splines to identify synapormorphies supporting a particular phylogenetic hypothesis of piranha evolution. Systematic applications of geometric morphometrics have also been proposed elsewhere (Rohlf and Marcus, 1993). A few workers have statistically compared groups of taxa using these methods. Rohlf and Marcus (1993), Rohlf et al. (1996), and Zelditch and Fink (1995) regressed partial-warp scores onto centroid size, finding significant allometric relationships. Rohlf et al. (1996) compared groups of mole taxa using canonical variates analysis and MANOVAs.

Multivariate statistical analysis of par-

tial-warp scores offers a potentially powerful and underexploited means of documenting shape differences among taxa. Our study represents one of the first such statistical applications of these methods. Morphometric analyses have been used by taxonomists to justify synonymy and to recognize new species-level taxa (Daly, 1985). Here, we document the capacity of the thin-plate spline to identify subtle but informative variations in shape that are not apparent to the naked eye. These analyses thus endorse the recognition of sibling species within the nominal species *N. bebbianae* that traditional taxonomic approaches could not discern (Karren, 1972). We further document significant shape differentiation in 6 of 19 comparisons between allopatric beetle populations, despite using a very conservative critical α (Table 4). The successful use of the thin-plate spline in uncovering shape differences between even closely related populations (e.g., *Alnus* and *Salix* beetles) indicates that this method would also be useful in future studies of morphologically homogeneous species. Although molecular genetic approaches have recently gained favor as a means of distinguishing such taxa, this study suggests that the shape characters discernible by the spline may often be sufficiently evolutionarily plastic to allow differentiation and discrimination of even closely related populations and taxa; relative warps clearly distinguished *Alnus* and *Salix* beetle populations, but rapidly evolving mtDNA sequences did not (Funk, 1996).

Two advantages of the thin-plate spline are its capacity to discern shape differences and the potential to graphically illustrate shape variation. Visual depictions of positional variation at landmarks (Fig. 2) and of the major axes of shape deformation (Figs. 3–5) allow variable regions to be identified for further study. Patterns of variation might suggest the taxonomic utility of particular regions within a particular group, whereas nonrandom patterns of variation may offer clues to the functional importance of particular structures. In our study, such graphical depictions have illus-

trated the previously unrecognized taxonomic value of tubercle position (Fig. 4) and pointed to relative abdomen size as a means of understanding the (possibly adaptive) nature of sexual dimorphism in both size and shape in *Neochlamisus*.

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