

Molecular phylogenetic analysis of evolutionary trends in stonefly wing structure and locomotor behavior

Michael A. Thomas, Kathleen A. Walsh, Melisande R. Wolf, Bruce A. McPherson, and James H. Marden*

208 Mueller Laboratory, Department of Biology, Pennsylvania State University, University Park, PA 16802

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Insects in the order Plecoptera (stoneflies) use a form of two-dimensional aerodynamic locomotion called surface skimming to move across water surfaces. Because their weight is supported by water, skimmers can achieve effective aerodynamic locomotion even with small wings and weak flight muscles. These mechanical features stimulated the hypothesis that surface skimming may have been an intermediate stage in the evolution of insect flight, which has perhaps been retained in certain modern stoneflies. Here we present a phylogeny of Plecoptera based on nucleotide sequence data from the small subunit rRNA (18S) gene. By mapping locomotor behavior and wing structural data onto the phylogeny, we distinguish between the competing hypotheses that skimming is a retained ancestral trait or, alternatively, a relatively recent loss of flight. Our results show that basal stoneflies are surface skimmers, and that various forms of surface skimming are distributed widely across the plecopteran phylogeny. Stonefly wings show evolutionary trends in the number of cross veins and the thickness of the cuticle of the longitudinal veins that are consistent with elaboration and diversification of flight-related traits. These data support the hypothesis that the first stoneflies were surface skimmers, and that wing structures important for aerial flight have become elaborated and more diverse during the radiation of modern stoneflies.

Insect flight is an example of a complex trait whose origin is difficult to explain by using a model that depends on gradual progression through intermediate stages (1, 2). How can tiny wings, simple wing hinges, and weak muscles provide a functional advantage over no wings at all? A novel solution to this riddle was recently provided by the discovery of surface skimming, a nonflying form of aerodynamic locomotion used by certain stoneflies (Plecoptera) and mayflies (Ephemeroptera) to move in two dimensions across water surfaces (3–7). By flapping their wings or by using them as nonflapping sails while their weight is supported by water, skimmers can achieve effective aerodynamic locomotion even with small wings and weak flight muscles (3, 4).

Surface skimming is now widely accepted as a plausible mechanical model for flight evolution (8–11), but there is considerably less support for the suggestion (3, 4) that skimming in modern stoneflies is a retained ancestral trait. Many pterygote insects have lost the ability to fly, including numerous stonefly species that are wingless or possess greatly reduced wings. There also appears to have been an evolutionary reduction in the number of cross veins in the wings of stoneflies in the superfamily Nemouroidea (12), the clade in which skimming was first described. Cross veins are structural elements that link the main longitudinal veins; in some locations they stiffen the wing, whereas in others they contribute to active and passive deformations of the wing planiform that enhance aerodynamic performance (13, 14). The stonefly taxa that are traditionally thought to be the basal group have wings with abundant cross veins, as do other basal pterygotes (mayflies, dragonflies, and various extinct fossil lineages), thereby suggesting that particular lineages of more recently evolved stoneflies have undergone an

evolutionary reduction in wing structural complexity. Surface skimming and reduced wing complexity may have evolved as correlated traits during an evolutionary reduction in flight proficiency in certain lineages of modern stoneflies.

To determine the evolutionary history of stonefly skimming and wing structural complexity, it is necessary to examine how these traits are distributed across the plecopteran phylogeny. This type of analysis has already been attempted for surface skimming (15), which resulted in the conclusion that skimming behavior is most likely a derived, apomorphic condition, i.e., a relatively recent loss of flight. However, that analysis had two serious shortcomings. First, the morphological characters used to construct that phylogeny were not compared with homologous traits of outgroup taxa to determine their polarity (ancestral vs. derived; ref. 16). Polarities were assigned based on resemblance to assumed ancestral conditions. This is problematic, and the resulting tree has limited utility for assessing evolutionary history. Second, the analysis assumed that skimming was restricted to the single species in which the behavior had been originally described (3), despite the fact that presence or absence of skimming in other stonefly taxa had not been determined. Lacking even a rudimentary knowledge of the taxonomic distribution of the trait in question, the analysis and conclusions are questionable.

Here we reexamine this question by using a rooted phylogenetic analysis based on DNA sequence data from stoneflies and a number of outgroup taxa. Mapping wing structural data and skimming behavior onto this phylogeny allows us to test hypotheses about the evolutionary direction of locomotor behavior and wing structural complexity.

Methods

Phylogenetic Analysis. The small subunit rRNA (18S) gene was sequenced for 34 stonefly species representing all families of Plecoptera (GenBank accession nos. AF311439–AF311472; a complete list of taxa and collection information can be found in Table 1, which is published as supplemental data on the PNAS web site, www.pnas.org). An additional stonefly sequence (*Mesoperlina pecirai*; Perlodidae) was obtained from GenBank (accession no. U68400). DNA was extracted by using standard phenol/chloroform protocols for alcohol-preserved material (17). Amplification of the 18S gene by the PCR used two oligonucleotide primers, rev18G (5'-AGGGCAAGTCTGGT-

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Abbreviations: NJ, neighbor joining; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF311439–AF311472).

*To whom reprint requests should be addressed. E-mail: jhm10@psu.edu.

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GCCA) and 18L (5'-CACCTACGGAAACCTTGTTAC-GACTT), generating an approximately 1,300-nt fragment.

Sequencing primers included the two PCR primers and two internal primers 18H (5'-TCAATTCCTTTAAGTTTGAGC) and rev18H (5'-GCTGAAACTTAAAGGAATTGA), which generated sequence fragments of approximately 700 nucleotides in length. Cycle-sequencing reactions were performed by using 3' BigDye-labeled dideoxynucleotide triphosphates and run on an Applied Biosystems Prism 377 DNA Sequencer. Raw data were analyzed by using the DNA STAR SEQMAN II sequence analysis program (DNASTar, Madison, WI).

Outgroup taxa used to root the phylogeny were selected by using a relative apparent synapomorphy analysis (RASA; ref. 18), which identifies outgroups that maximize the ratio of informative phylogenetic signal to uninformative noise, thereby increasing the probability that the correct phylogeny is recovered. Of the outgroup combinations we tested (a list can be found in Supplemental Table 2 published on the PNAS web site, www.pnas.org), a diverse set of Hemiptera, Orthoptera, Dermaptera, Phasmatodea, Embioptera, Grylloblattodea, and Blattodea (GenBank accession nos. U06478, U06480, U09207, U06477, Z97573, Z97574, Z97594, Z97561, Z97575, Z97593, Z97569, and Z97592) yielded the highest t_{RASA} statistic ($t_{\text{RASA}} = 31.814$; $P < 0.001$).

Sequences were aligned by using the CLUSTAL W alignment model (19). The alignment consisted of 1,696 sites, including gaps. A total of 578 sites, primarily in one large hypervariable region, were unalignable and therefore were excluded from our analyses (sites 122–510, 591–600, 662–670, 774–776, 1182–1240, 1353–1362, 1564–1608, and 1644–1696). The analyzed data set consisted of 331 variable (133 nonparsimony informative and 198 parsimony informative) and 787 constant sites. Average nucleotide frequencies estimated by PAUP were 24:23:28:25 (A:C:G:T). There was no significant deviation from these frequencies among taxa (χ^2 test for all sites and for variable sites). A likelihood ratio test of the hypothesis that base frequencies were equal was not rejected ($P = 0.60$) by the MODELTEST program [(20); $-\ln L$ values were calculated by PAUP]. The average transition-to-transversion ratio (Ti/Tv) was 1.88, estimated by the Kimura 2-parameter model. A likelihood ratio test of the hypothesis that Ti = Tv was rejected ($P < 0.0001$). Given the base composition, substitution rates, and low average distance in our data set (average Jukes–Cantor distance < 0.1), we followed the suggestion of Kumar *et al.* (21) that the Kimura 2-parameter model would be most appropriate.

Phylogenetic analyses were accomplished by using PAUP*4.0B4A (22). We approximated the Kimura 2-parameter model to construct a neighbor joining (NJ) tree and to find the minimum evolution (ME), and maximum likelihood (ML) trees. For all analyses, we used pairwise deletion and included all substitutions (transitions and transversions). We used heuristic searches to find trees based on maximum parsimony (MP), ME, and ML criteria. Support for the NJ, ME, and MP trees was measured by using the bootstrap method with 1,000 replicates; for the more computationally intensive ML trees, we used 100 bootstrap replicates.

Locomotor Behavior. We have examined the locomotor behavior of 23 species of stoneflies from 11 families on 4 continents (North America, South America, Europe, and Australia). Descriptions of these behaviors and the methods used to examine skimming are published elsewhere (3–5, 7). High-speed video recordings (500 frames per second) of distinct forms of surface skimming are available at <http://www.bio.psu.edu/People/Faculty/Marden/PNASFig2.html>.

Wing Structure. To assess wing structural complexity, we counted the number of cross veins between the costal vein and either the

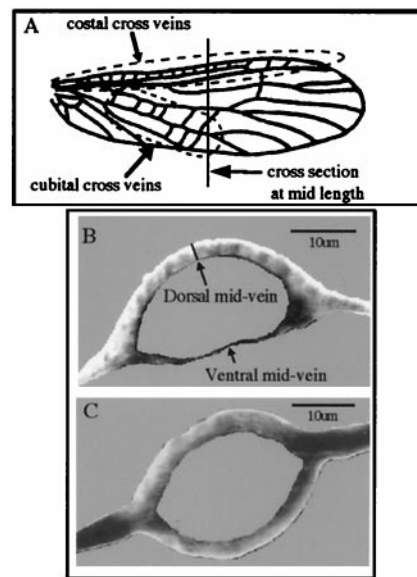


Fig. 1. (A) Regions of stonefly wings from which cross veins were counted and location of the chordwise cross section used for measurements of vein morphology. (B and C) Examples of cross sections of longitudinal wing veins and the measurements taken for cuticle thickness. B shows the medial vein of a relatively basal species, *Taeniopteryx burksi*; C shows the medial vein of the relatively derived species, *Pteronarcella badia*.

subcostal or radial vein (hereafter referred to as costal cross veins) and those between the anterior cubital vein and both the medial vein and the posterior cubital vein (hereafter referred to as cubital cross veins; Fig. 1A). To examine wing-vein structure, we cut 1-mm-thick sections across the entire wing chord, perpendicular to the longitudinal axis of the wing at the point midway between the base and tip of the forewing (Fig. 1A). Thick sections were embedded in Spurr's resin, then sectioned ($0.5 \mu\text{m}$) by using an ultramicrotome equipped with a diamond knife. These sections were mounted on slides, sputter coated, and viewed under a scanning electron microscope (JEOL JSM 5400) to obtain an image of the cross section of each longitudinal vein, from which we measured the thickness of the dorsal and ventral cuticle at the midpoint of the vein (Fig. 1B). Our sample of specimens for wing-vein morphometric measurements came primarily from other scientists who had no knowledge of the hypotheses we were testing (i.e., no bias in choice of species).

Statistical Analyses of Wing Structural Complexity. We used square root transformations of wing length and vein thickness to achieve normality and homoscedasticity. To compare means of distributions that had significantly different variances, we used a nonparametric analysis of variance (Kruskal–Wallis test). To control for potential statistical nonindependence of data from related species, we used branch lengths from our NJ tree as estimates of evolutionary distance to generate independent contrasts (23, 24). This technique uses a Brownian motion model of phenotypic evolution along with trait differences between certain pairs of species and/or nodes on the tree to generate, for N species, a set of $N - 1$ standardized contrasts that are statistically independent.

Results

All of the phylogenetic analyses that we performed (NJ, MP, ME, and ML methods) indicated that the family Nemouridae is the basal plecopteran clade (e.g., the NJ tree in Fig. 2; bootstrap values for the node separating Nemouridae from the remainder of Plecoptera were 87, 81, 75, and 54 for the NJ, ME, MP, and

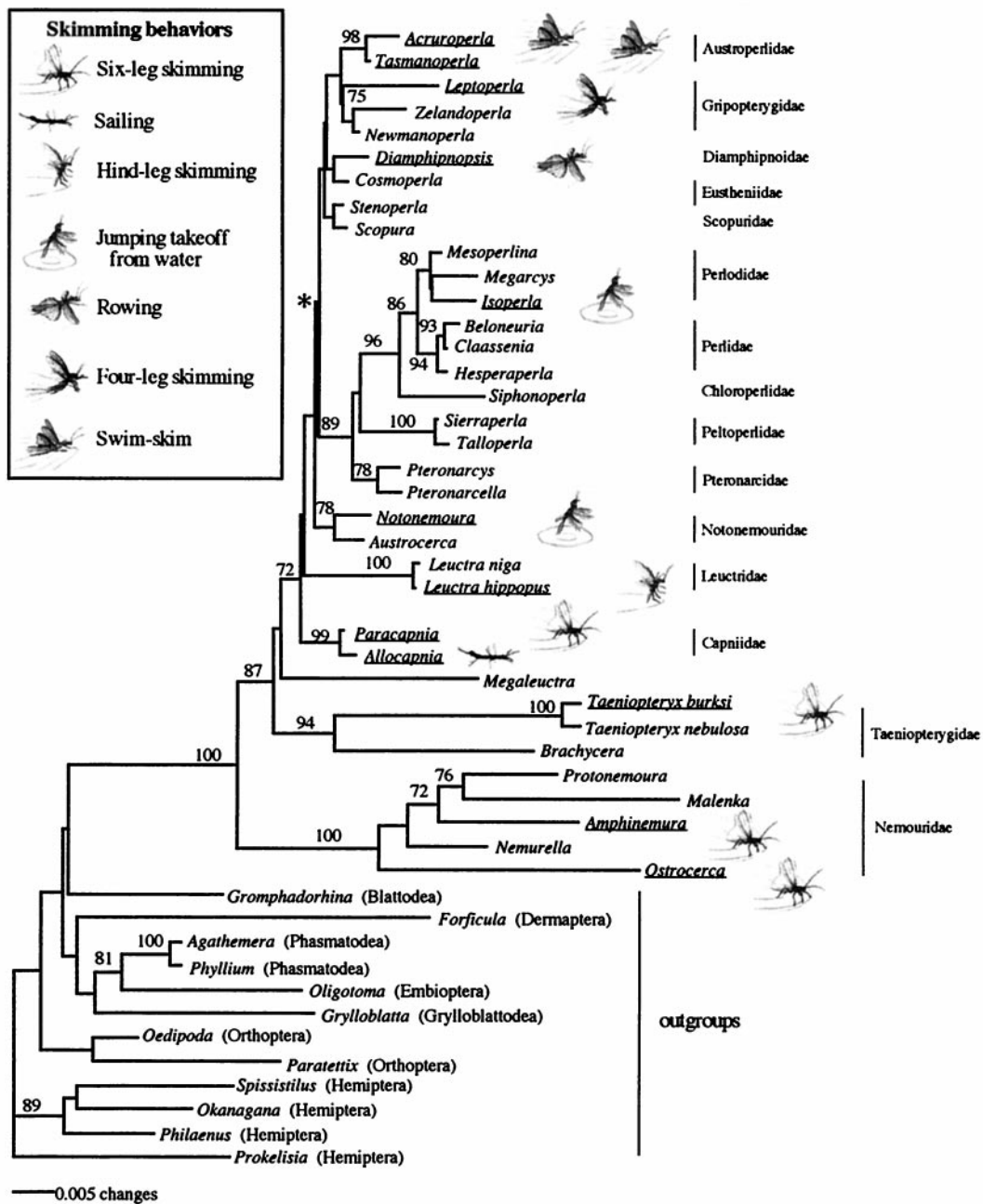


Fig. 2. A phylogeny of the Plecoptera, constructed by using a NJ analysis with sequence data from the 185 gene. Taxa below *Ostrocerca* are outgroups. Numbers on the left of nodes indicate bootstrap support (1,000 replicates; only values over 70% are shown). The asterisk marks the node separating the superfamily Nemouroidea from the more derived taxa; although not well resolved by the 185 sequence data alone, this node has a bootstrap support value of 89 in a MP analysis that combines 185 sequence data with morphological character data. Underlined taxa are those included in our phylogenetic analysis that are known to be surface skimmers or that initiate flight by jumping from the water (3–7); taxa not underlined have not been sampled (except *Scopura*, which is wingless and therefore incapable of any form of winged locomotion, and the Perlidae, which we have never observed to skim). Icons to the right of each underlined taxon show the type of behavior used by that species. A version of this figure containing links to video recordings of the behaviors is available at <http://www.bio.psu.edu/People/Faculty/Marden/PNASFig2.html>.

ML trees, respectively). Robust support for the basal status of Nemouridae allowed us to designate this family as an outgroup for the rest of the Plecoptera, then use a MP analysis of our molecular data together with morphological characters (data from ref. 16, with recoding of nonindependent characters according to ref. 15) to obtain better resolution of relationships among the plecopteran families. One important result of that analysis was strong support (bootstrap value of 89) for the node (asterisk in Fig. 2) that separates the superfamily Nemouroidea

(Nemouridae + Taeniopterygidae + Megaleuctridae + Capniidae + Leuctridae + Notonemouridae) from the remainder of the Plecoptera. This topology is a significant departure from previous phylogenies constructed by using only morphological data and assumed character polarities (15, 16); those studies place the Nemouridae and the rest of the Nemouroidea as a relatively derived clade. Aside from rooting, the topologies that we obtained by using either molecular or a combination of molecular and morphological data are congruent with topologies

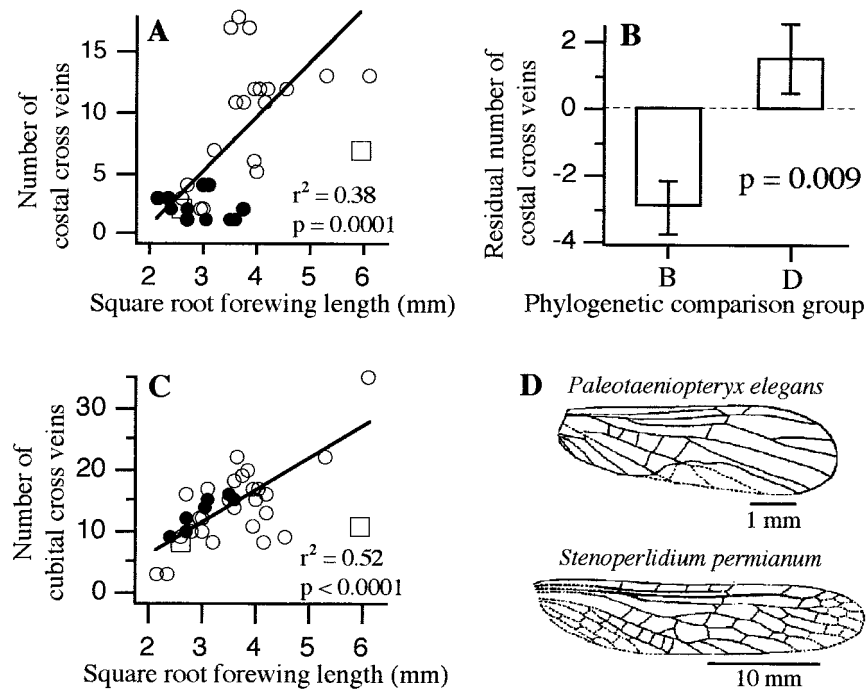


Fig. 3. (A) Number of costal cross veins in the forewing of 34 species of stoneflies from 13 families. Filled circles represent taxa from the basal superfamily Nemouroidea, open circles represent taxa from the more derived clades; squares represent fossil species (see D; these were excluded from the statistical analyses). (B) Mean residual (+SE) number of costal cross veins in forewings of the two basal clades ("B") vs. the more derived clades ("D"; $P = 0.009$); residuals are from the regression of costal cross vein number on wing length. (C) Number of cubital cross veins as a function of wing length. There was no significant difference in residual values for cubital cross veins in basal vs. derived taxa ($P = 0.39$). (D) Drawings of the earliest known fossil stonefly wings [≈ 260 million years ago; reproduced with permission from ref. 12 (Copyright 1965, Annual Reviews)].

that have been obtained from analyses based solely on morphological data (i.e., a monophyletic clade of pteronarcids + peltoperlids + chloroperlids + perlids + perlodids; a clade of eustheniids + diamphipnoids + gripopterygids + austroperlids; the affinity of taxa within the superfamily Nemouroidea).

Surface skimming behavior is distributed widely across the phylogeny (Fig. 2; skimming in an additional 10 stonefly species beyond those included in our phylogeny is reported in ref. 7), including the basal Nemouridae and other families within the Nemouroidea. Flight capability is also widely distributed, as most stoneflies that use skimming can also fly, albeit quite weakly in comparison to the vast majority of other insects (3, 5, 7). Thus, our data indicate that the ancestral condition for Plecoptera was most likely a combination of relatively weak flight and surface skimming.

To investigate the evolutionary history of stonefly wing design, we first examined the data for directional trends in the mean number of costal and cubital cross veins. Because this analysis seeks to determine whether there is a phylogenetic pattern in mean trait values, we treated species as replicate samples and used conventional statistical analyses to evaluate the presence or absence of a phylogenetic trend. Our data indicate that species in the basal Nemouroidea have fewer costal cross veins than the more derived taxa, both before and after adjusting for wing length (Fig. 3A and B). No such directional trend is evident for length-adjusted differences in the mean number of cubital cross veins (Fig. 3C).

We also examined wing morphology in a way that corrects for potential statistical nonindependence caused by shared evolutionary history (i.e., the phylogenetic trends detected above). Techniques such as independent contrasts (ICs; refs. 23 and 24) essentially remove phylogenetic trends and can be used here to examine the variability of traits around the phylogenetically

adjusted mean. Using ICs for vein number and wing length, we found no significant relationship between the number of costal cross veins and wing length ($r^2 = 0.15$; Fig. 4A), but there was significantly more variability in costal cross-vein number at nodes within the more derived clades than nodes within the basal superfamily Nemouroidea (Bartlett's test of homogeneity of variances; $P = 0.02$). The number of cubital cross veins was positively related to wing length in the IC comparison (Fig. 4B; $r^2 = 0.42$; $P = 0.002$), and residuals from that regression also showed more variability within the more derived clades than within the Nemouroidea ($P = 0.03$). These analyses indicate that, in addition to the directional trend in costal cross-vein number

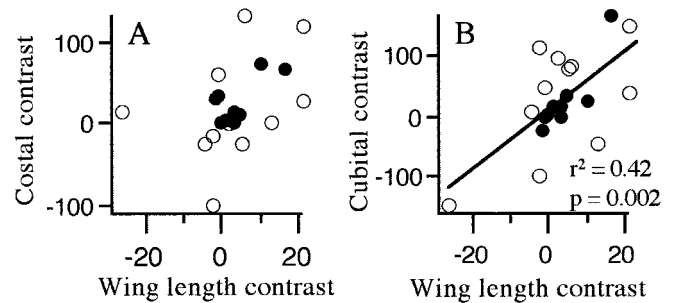


Fig. 4. Plots of standardized independent contrasts derived for the number of costal (A) and cubital (B) cross veins. Closed circles represent values from nodes within the basal superfamily Nemouroidea; open circles represent values from nodes within the more derived clades. Sample sizes ($n = 21$ species) are reduced in these plots compared with Fig. 3, because not all species sampled for wing veins were included in the phylogenetic analysis; points shown here represent only those taxa for which we also obtained measures of phylogenetic distance.

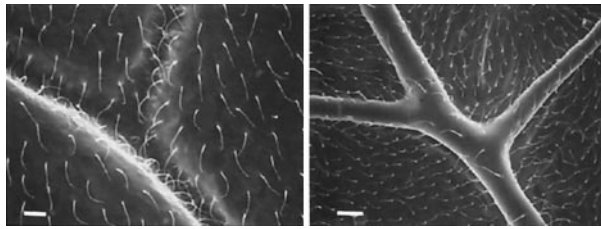


Fig. 5. Scanning electron microscope images of the ventral surface of the forewing of (Left) *Taeniopteryx burksi* (Taeniopterygidae; bar = 10 μm) and (Right) *Paragnetina media* (Perlidae; bar = 50 μm). Exposure to a vacuum causes the collapse of the vein cuticle over the entire ventral surface of *T. burksi* wings; no such collapse occurs in *P. media*, which has thicker ventral vein cuticles. There is no vein collapse on the dorsal surface of wings from either species (image not shown).

shown above, stoneflies have also undergone a diversification (i.e., radiation) in the number of both costal and cubital cross veins.

The earliest known stonefly fossils corroborate the hypothesis that cross veins have become more numerous in the relatively derived modern taxa. Cross-vein abundance in the forewing of a relatively basal fossil species, *Paleotaeniopteryx elegans* [(12); \approx 260 million years ago; Taeniopterygidae; Fig. 3D], does not differ from modern Nemouroidea species (Fig. 3A and C), whereas the relatively derived fossil species, *Stenoperlidium permianum* [(12); Eustheniidae; Fig. 3D], has a reduced number of both costal and cubital cross veins in comparison with similar-sized modern species (Fig. 3A and C). Thus, the abundant cross veins that typify modern members of the family Eustheniidae are apparently a relatively recent adaptation, perhaps in response to greater reliance on flight.

Cross-sectional structure of the longitudinal wing veins also shows interesting evolutionary patterns. Our interest in vein structure (Fig. 1B and C) was stimulated by the observation that all of the wing veins over the entire ventral wing surface of the relatively basal skimmer *Taeniopteryx burksi* collapsed when the wings were exposed to a vacuum, whereas those of a more derived nonskimming perlid stonefly did not (Fig. 5). These differences are not the result of a directional evolutionary trend, because the mean thickness (after adjusting for wing length) of the cuticle at the ventral and dorsal midpoint of the longitudinal wing veins does not differ between taxa within the Nemouroidea vs. the more derived taxa (Fig. 6; $P = 0.37$ and 0.73 for ventral

and dorsal, respectively). However, analyses of independent contrasts indicate that skimming species have significantly thinner vein cuticles (Fig. 6; $P = 0.038$ and 0.003 , respectively, for one-tailed tests of length-adjusted contrasts of ventral and dorsal cuticle thickness) than do taxa belonging to families in which we have never observed skimming (Perlidae, Notonemouridae) or which skim very poorly (Chloroperlidae and Perlodidae, which maintain skimming for only a few wing strokes). These analyses indicate that the design of the tubular vein structures (Fig. 1B and C) has responded to patterns of wing usage.

Discussion

Rooted phylogenies based on 18S rDNA sequence data, and a combination of molecular and morphological data, provide strong support for the hypothesis that the superfamily Nemouroidea is the basal clade within the Plecoptera. A number of species that we have examined in this superfamily, including species in the most basal family Nemouridae, use the same method of moving in two dimensions on the surface of water; they flap their wings through an arc of $90\text{--}110^\circ$ while their legs are spread in a stereotypical stance, with all six tarsi in continuous contact with the water surface [i.e., six-leg skimming (3, 5, 7)]. Most of the Nemouridae are flight capable or at least marginally so, and this appears to be true for most species of stoneflies. Surface skimming and various levels of flight ability/inability also occur in gripopterygid, austroperlid, and diampiphoid stoneflies (7), which occupy a relatively derived position on the phylogenetic tree. The widespread distribution of skimming among basal and derived taxa indicates that skimming, along with weak flight, is likely to be a retained ancestral trait. An alternative hypothesis, that skimming is a recent loss of flight in a restricted set of taxa (15), is not supported.

Surface skimming places far less stringent mechanical demands on the wings than does flying, because contact with the water provides weight support. Reduced mechanical demands should allow successful surface skimming with structurally simpler wings, and our analyses of vein number and cross-sectional structure support this hypothesis. Our data indicate that the number of costal cross veins has become greater during the radiation of Plecoptera, and that there has been an increase in variability in the number of both costal and cubital cross veins. Phylogenetically independent contrasts show that the cuticle that forms the longitudinal wing veins is thicker among stoneflies that only fly and have apparently lost the ability to skim. Fossil species such as *Stenoperlidium permianum* (Fig. 3D), whose wings had a reduced number of cross veins, may have been

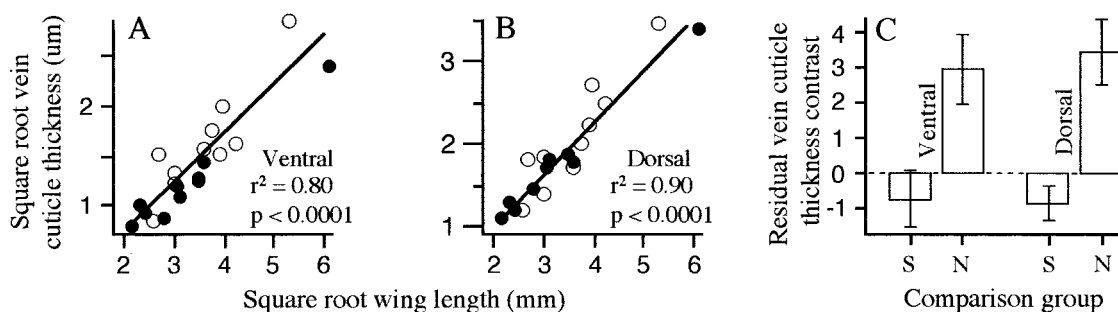


Fig. 6. (A and B) Relationship between thickness of the cuticle of longitudinal wing veins (mean of all longitudinal veins from within each individual; total sample, 147 veins from 21 species) and wing length. Filled circles represent species that skim; open circles represent species belonging to families in which we have never observed skimming (Perlidae, Notonemouridae) or which skim only transiently (i.e., for only a few wing strokes; Chloroperlidae, Perlodidae). (C) Mean residuals (\pm SE) from regressions of phylogenetically independent contrasts of vein cuticle thickness and wing length. Mean residuals for vein thickness differ significantly for nodes within clades of skimmers (S) vs. nonskimmers (N) in the comparison of both the ventral and dorsal measurements ($P = 0.038$ and 0.003 respectively; one-tailed tests). Sample sizes are reduced in these plots compared with Fig. 3 because not all species sampled for vein thickness were included in the phylogenetic analysis; data used here for independent contrasts represent only those taxa ($n = 11$ species) for which we also obtained measures of phylogenetic distance.

flight-incapable skimmers, or perhaps they flew less frequently or with less acceleration and maneuverability. This interpretation fits nicely with the hypothesis that predation by modern surface-feeding fish makes skimming a dangerous form of locomotion for most extant species (6, 7) and thus a largely obsolete behavior that is now used primarily for emergency escape from accidental contact with water. Winter stoneflies (taeniopterigids and capniids), which are active during seasons when fish do not feed at the surface, make routine use of skimming (3–4), as do certain flightless mayflies (Ephemeroptera) that inhabit rivers in Madagascar that lack insectivorous fish (6). For stoneflies and insects in general, a gradual increase in the intensity of predation at the water surface may have driven a radiation away from routine use of skimming.

Allocapnia stoneflies (Capniidae) skim by sailing; they raise their wings in response to wind and are incapable of flapping. Because this behavior is mechanically simpler than flapping, it was originally proposed that sailing might be the ancestral condition (4). However, a behavioral survey (7) has shown that another capniid (*Paracapnia angulata*) uses wing flapping six-leg skimming in a manner identical to nemourids and taeniopterigids. Similarities between the leg and body postures of sailing *Allocapnia* and six-leg skimmers, along with the derived phylogenetic position of capniids in relation to six-leg skimming nemourids, suggest that sailing evolved when six-leg skimmers lost the ability to flap but retained other features of their skimming behavior. Modifications of six-leg skimming can also explain the other forms of skimming that we have observed (Fig. 2), based on postural changes that either increase (rowing, swim-skim) or decrease (four-leg and hind-leg skimming) contact with the water.

Our present study provides support for the hypothesis that surface skimming has deep evolutionary roots within stoneflies. However, the greater challenge remains to determine whether skimming was a transitional stage leading to flight in winged insects as a whole. An alternative hypothesis is that the immediate ancestors of stoneflies had secondarily reduced wing structures and flight ability, and that although skimming and relatively simple wings are ancestral in stoneflies, these traits are unrelated to morphology and behavior of other winged insects. It is not presently possible to distinguish between these com-

peting hypotheses, but there is a diverse and growing body of evidence for a progressive evolution of flight from aquatic origins. Analyses of fossils indicate that wings evolved from moveable gills of aquatic ancestors [(25–27); such gills are present in certain taxa of modern stoneflies and mayflies (<http://www.bio.psu.edu/People/Faculty/Marden/movies/gillflap.mov>)]. The wings-from-gills hypothesis is supported by molecular genetic analyses of wing development (28, 29), the types of sensory receptors on wings (30), and phylogenetic studies, which show that insects and crustaceans are sister taxa (31–36). Both the anatomical and phylogenetic data point to an aquatic or semiaquatic setting for wing origins. Early winged insects diversified into two main clades, the Neoptera (of which stoneflies are a relatively basal group) and the Paleoptera, which are represented in modern forms by mayflies (Ephemeroptera) and dragonflies (Odonata). Like stoneflies, all extant Paleoptera have aquatic immature stages, deposit their eggs on or near the surface of water, and, with the exception of modern dragonflies, have very similar water-resistant hairs on their wings (3, 37). A recent examination of wings and thoraces of fossil mayflies from the Carboniferous and Permian revealed that they appear to have had greatly reduced flight ability (14). Thus, mayflies have undergone an elaboration of flight-related traits that is, in its general features, parallel to what our analyses suggest has occurred in stoneflies. A reasonable hypothesis to account for this diverse body of observations is that the first winged insects were surface skimmers.

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