

being more tractable by deterministically describing the dynamics of a population density $n(x,u)$, these approximations are problematical. First, such continuum approximations are based on the limit of infinite local population sizes (local both in x and u), which is even more difficult to justify biologically than the limit of infinite global population size, widely used in population ecology. Second, a conveniently simple reaction-diffusion approximation of this system, derived for small σ_m and σ_s , is dynamically unstable. Third, these approximations ignore the implications of reproductive (and other) pair correlations and local density fluctuations, both of which have been shown to critically affect ecological and evolutionary dynamics³⁰. Fourth, the deterministic approximation blurs the sharp bifurcation boundary in Fig. 3a and is also inaccurate in predicting the boundary's location. Fifth, extending the deterministic approximation to multilocus genetics is not feasible without incurring further unjustified assumptions.

Received 12 July; accepted 18 October 2002; doi:10.1038/nature01274.

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Acknowledgements We thank H. Metz, D. Tautz, G. Meszóna, D. Schluter, E. Knox, O. Leimar, M. Kirkpatrick and T. Barraclough for discussions and comments. The order of authors is reverse alphabetical.

Competing interests statement The authors declare that they have no competing financial interests.

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Loss and recovery of wings in stick insects

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The evolution of wings was the central adaptation allowing insects to escape predators, exploit scattered resources, and disperse into new niches, resulting in radiations into vast numbers of species¹. Despite the presumed evolutionary advantages associated with full-sized wings (macroptery), nearly all pterygote (winged) orders have many partially winged (brachypterous) or wingless (apterous) lineages, and some entire orders are secondarily wingless (for example, fleas, lice, grylloblattids and mantophasmatids), with about 5% of extant pterygote species being flightless^{2,3}. Thousands of independent transitions from a winged form to winglessness have occurred during the course of insect evolution; however, an evolutionary reversal from a flightless to a volant form has never been demonstrated clearly for any pterygote lineage. Such a reversal is considered highly unlikely because complex interactions between nerves, muscles, sclerites and wing foils are required to accommodate flight⁴. Here we show that stick insects (order Phasmatodea) diversified as wingless insects and that wings were derived secondarily, perhaps on many occasions. These results suggest that wing developmental pathways are conserved in wingless phasmids, and that 're-evolution' of wings has had an unrecognized role in insect diversification.

Stick insects are large terrestrial insects that exhibit extreme forms

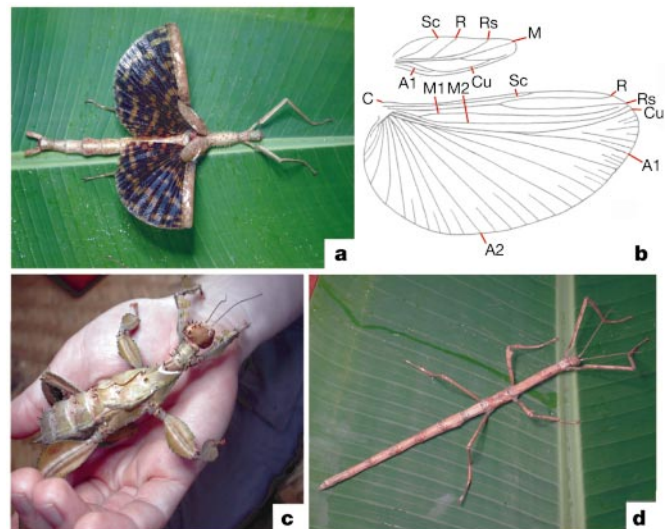


Figure 1 Examples of wing features in stick insects. **a**, Example of a fully winged (macropterous) female phasmid (*Phasma gigas*) with enlarged hindwings and thickened forewings. **b**, Wing venation of male *Phyllium celebicum* with major veins labelled, demonstrating homology with other insect wing veins. A, anal vein; C, costa vein; Cu, cubitus vein; M, medial vein; R, radius vein; Rs, radial sector vein; Sc, subcosta vein. **c**, Example of a partially winged (brachypterous) female phasmid (*Extatosoma popa*) with reduced hindwings. **d**, Example of a wingless (apterous) female phasmid (*Leprocaulinus* sp.) with wings entirely absent.

of morphological and behavioural crypsis as mimics of sticks and leaves. Phasmids are chiefly arboreal insects, but a few of the more robust taxa (for example, *Eurycantha*, *Agathemera*, Heteropterygidae) occur primarily near the ground, in the leaf litter. The monophyly of Phasmatodea is supported by a series of distinctive morphological characters including prothoracic repellent glands, absence of mitochondria in spermatozoa, and male vomer^{5,6}. Phasmids belong among the basal winged insect orders (Polyneoptera), but their sister group is unknown, although Caelifera (grasshoppers)⁷, Orthoptera⁸, Dermaptera (earwigs)⁶, Grylloblattoidea

and Dermaptera⁹, Dictyoptera (mantids, cockroaches and termites)¹⁰, and Embiidina (web spinners)^{6,11} are all candidates. It is difficult to decipher the phylogenetic relationships within Phasmatodea because of the convergent morphology associated with their remarkable crypsis. Current classification is based on some dubious morphological characters¹², and no formal investigation of phasmid phylogeny has yet been published.

Of the 3,000 described species of phasmids placed in 3 families and approximately 500 genera, only 40% are fully winged, with the remainder being partially winged or wingless. In fully winged

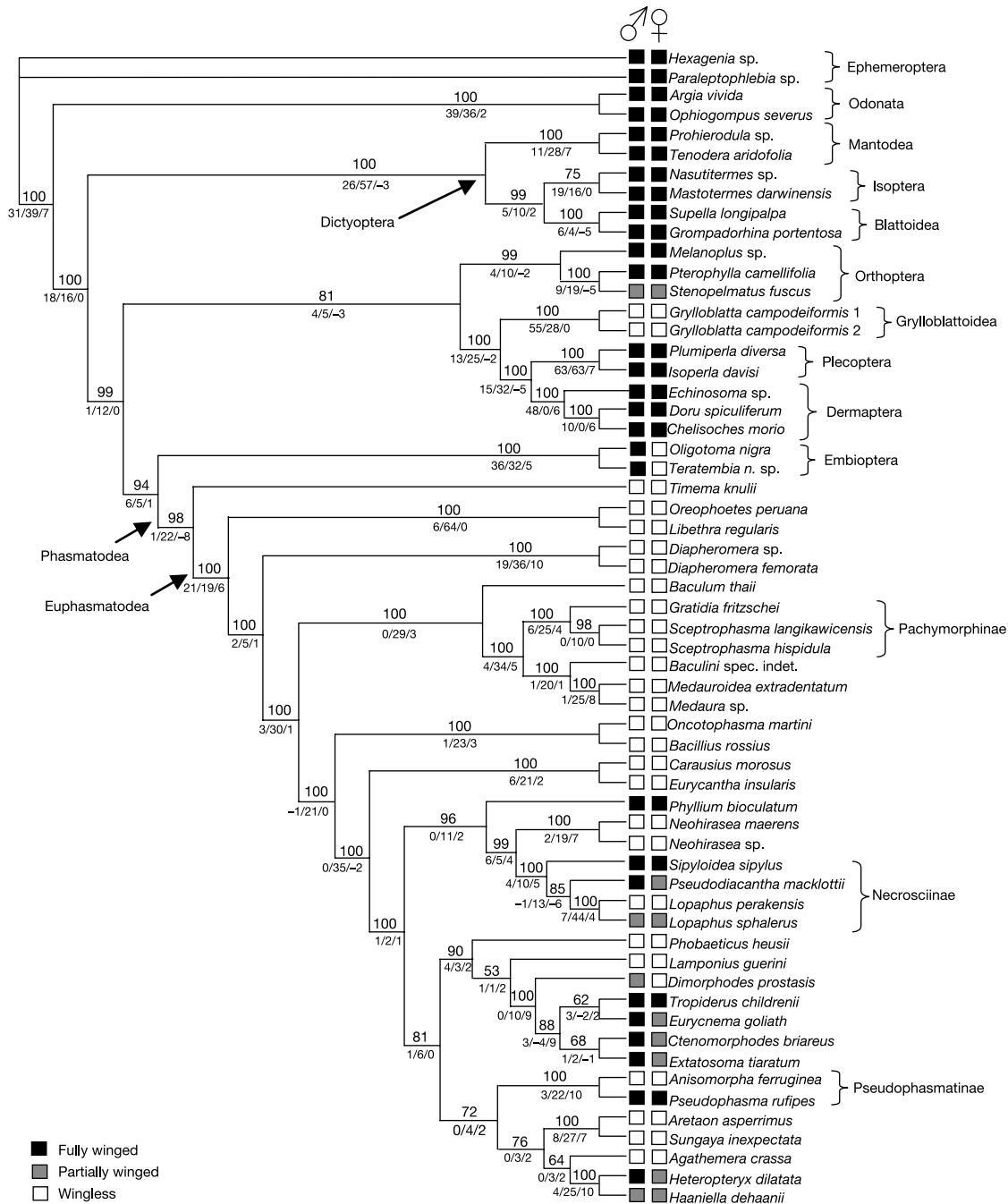


Figure 2 Phylogeny of Phasmatodea on the basis of molecular data. Shown is the single optimization alignment tree based on 18S rDNA, 28S rDNA and histone 3. Nonparametric bootstrap supports are given above each node and partitioned Bremer supports are below each node in the order 18S/28S/H3. This topology is congruent with the maximum

likelihood and bayesian topology (see Supplementary Information). Boxes at the end of nodes represent wing character states for males and females, respectively. n. sp., new species; spec. indet., undetermined species.

phasmids, the front wing is reduced and thickened, and the hind wing is enlarged with an extensive anal region (Fig. 1a). Vein homology is readily assessed between phasmids and other insect groups, and analysis indicates that phasmid wings are homologous to those of other insects (Fig. 1b). Partially winged phasmids have reduced hindwings with a small anal region, and individuals are not capable of sustained flight (Fig. 1c). In wingless phasmids, no wing remnants are present (Fig. 1d). The extent of wing development of male phasmids is never less than females: if the male is wingless, the female is always wingless; if the male is partially winged, the female is partially winged or wingless, but never fully winged; if the male is fully winged, the female may be fully winged, partially winged or wingless.

DNA sequence data were used to place the Phasmatodea among the polyneopterous insect orders and to estimate phylogenetic relationships for major stick insect lineages. The entire regions of 18S ribosomal DNA (18S rDNA; about 1,900 base pairs (bp)), 28S rDNA (2,250 bp), and a portion of histone 3 (H3, 372 bp) were sequenced from 22 outgroup and 37 ingroup taxa representing all Polyneoptera and 14 of the 19 recognized phasmid subfamilies. Trees were reconstructed through optimization alignment and nodal support determined by nonparametric bootstrap and partitioned Bremer support (see Methods and Fig. 2). Partitioned Bremer supports indicate that 31% of the signal is provided by 18S, 62% by 28S and 7% by H3. Overall, H3 provides resolution at the shallow nodes, 18S at the deep nodes, and 28S throughout the

entire topology. Most of the nodes are very well supported, with 44 out of 56 nodes with bootstrap values of 95 or higher and 52 out of 56 nodes with a total Bremer support of 5 or greater. These data support the monophyly of each insect order, the basal placement of Timematidae, and the monophyly of the groups Euphasmatodea, Pachymorphinae, Necrosociinae, Pseudophasmatinae and Lanceocercata, in agreement with other studies^{5,6,8,13}. Analyses using maximum parsimony, maximum likelihood, and bayesian methods result in a topology that is highly congruent with the optimization alignment topology (see Supplementary Information).

The most parsimonious reconstruction (MPR) of the states 'winged' and 'wingless' unambiguously places the ancestral state for phasmids as wingless, with wings derived on four (ACCTRAN optimization) or five (DELTRAN optimization) occasions (Fig. 3). Forcing the ancestral state as winged requires 13 steps in males and 14 steps in females, and is the MPR only when wing gain is weighted six times wing loss. Similar results are found when winged is divided into 'fully winged' and 'partially winged' and the states are treated as unordered. Wing states were also mapped by means of likelihood methods on both the bayesian and likelihood trees, to take into account branch lengths in determining probabilities of ancestral states¹⁴. When the rate of wing gain is set equal to that of wing loss, the phasmid ancestor is reconstructed as wingless ($P < 0.001$), with four independent wing gains in more derived stick insect lineages. When the ratio of the rate of wing loss over wing gain ranges from 1 to 1,400, there is a >95% probability that the ancestral phasmid was wingless.

These results support the hypothesis that the ancestral condition in Phasmatodea is wingless, that the first six basal phasmid lineages are entirely wingless, and that fully developed wings were derived later in phasmid evolution, on as many as four occasions. Clearly, the presence of wings is a very plastic feature in phasmids, with congeneric species (for example, *Lopaphus*) exhibiting both partially winged and wingless states. One of the correlates with winglessness in insects is increased female fecundity^{2,3}, and as phasmids scatter specially modified eggs individually rather than concentrating them in large numbers similar to their sister taxon, there may have been a selective advantage early in phasmid evolution to shift to winglessness to facilitate fecundity and increased crypsis. The detailed homology in wing features shared among phasmids and other insects suggests that wings did not re-evolve *de novo* in phasmids, but are rather a re-expression of the basic insect wing which was lost in ancestral stick insects. Entomologists have long assumed that re-evolution of wings in apterous lineages was impossible, because functional wings require complex interactions among multiple structures, and the associated genes would be free to accumulate mutations in wingless lineages, effectively blocking the path for any future wing reacquisition. However, this assumption requires that developmental pathways for wing formation are largely independent of pathways required for development of other structures. For instance, in *Drosophila* and other insects, leg and wing imaginal discs have a common origin from a single group of cells and the developmental pathway for wing determination has been largely co-opted (recruited) from the pathway required for limb formation^{15,16}. Therefore it is not surprising that the basic genetic instructions for wing formation are conserved in wingless insects, because similar instructions are required to form legs, and probably other critical structures¹⁶. Studies of flight motor patterns in flying and non-flying phasmids indicate that the non-flying phasmids have retained the neural structures and basic functional circuitry required for flight, as indicated by flight-specific neural activity in thoracic muscles¹⁷, demonstrating that the loss of wings does not correlate with the loss of flight musculature and innervation. Wing development depends on multiple gene systems, transcription factors, secreted proteins, and receptors¹⁵, and mutations in any one of these factors may lead to winglessness. Given the multitude of factors involved in wing

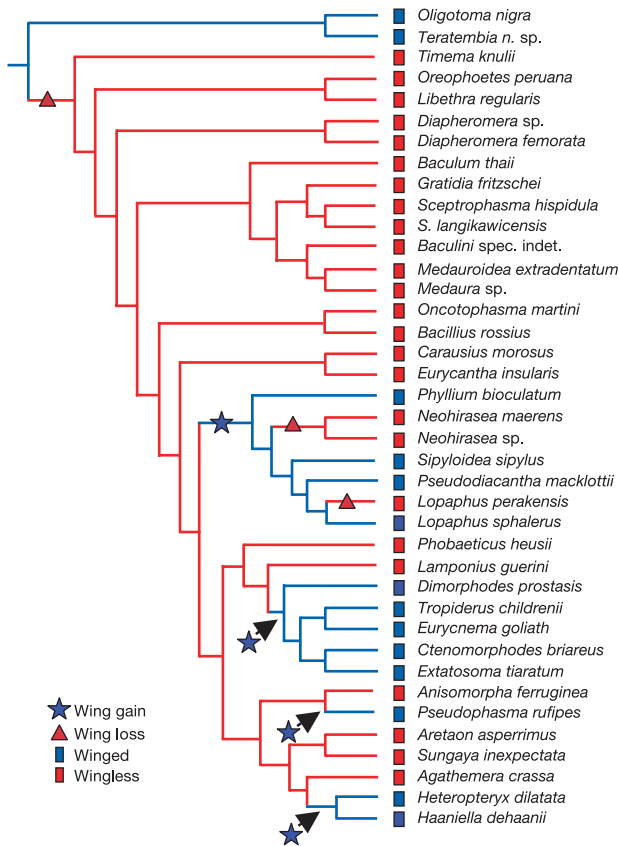


Figure 3 Character mapping of wing types on phasmid phylogeny. Parsimony optimization (ACCTRAN) of winged (blue) and wingless (red) states for male phasmids on the optimization alignment topology. This reconstruction requires seven steps with four wing gains and three losses; DELTRAN optimization requires five wing gains and two losses. Maximum likelihood reconstruction produces similar results (see Supplementary Information).

formation, it seems probable that the specific cause for winglessness will differ from lineage to lineage, but that the basic blueprint for wing formation can remain largely intact, even over large evolutionary time periods.

Re-evolving complex structures from the same basic building blocks may be a more general trend in evolution than previously recognized. For example, it is plausible that all modern animals with eyes evolved from a common ancestor that possessed a primitive image-forming organ¹⁸, controlled by the master gene *Pax-6*. Our results support the hypothesis that the developmental pathway for wing formation evolved only once in insect diversification, but that wings evolved many times by silencing and re-expressing this pathway in different lineages during insect evolution. Thus, wing loss does not seem to be an evolutionary dead end, and the ability to regain a wing over evolutionary time means that lineages have the adaptive advantages of being both winged and wingless. The transition from wingless to flying forms may be a common theme in insect evolution, as it has been suspected within one genus of water striders¹⁹, and may occur in cockroaches and Hemiptera, which also exhibit a wide diversity of wing forms. To our knowledge, this is the first example of a complex feature being lost and later recovered in an evolutionary lineage, and it is possible that the reacquisition of complex features may have an important role in evolutionary diversification. □

Methods

Taxon selection and molecular methods

Outgroup exemplars include multiple representatives of Ephemeroptera, Odonata and all polyneopterous insect orders, except for Zoraptera whose assignment to Polyneoptera is more questionable^{6,8}. Phasmid exemplars include all families and 14 out of 19 subfamilies¹², including Bacteriinae (1 sp.), Eurycanthinae (1 sp.), Diapheromerinae (5 spp.), Lonchodinae (3 spp.), Necrosiinae (4 spp.), Pachymorphinae (3 spp.), Phasmatinae (7 spp.), Tropidoderinae (2 spp.), Xeroderinae (1 sp.), Bacillinae (1 sp.), Heteropteryginae (4 spp.), Phyllinae (1 sp.), Pseudophasmatinae (3 spp.) and Timeminae (1 sp.). The five subfamilies left unsampled are mostly minor groups/species-poor taxa. Genomic DNA was extracted from ethanol-preserved specimens from leg muscle tissue using standard protocols¹¹. Genomic DNA templates and controls were amplified by polymerase chain reaction (PCR) in a Perkin-Elmer 9700 thermocycler using primers modified for insects^{20,21}. We monitored product yield, specificity, and potential contamination by agarose gel electrophoresis. Target products were purified and cycle-sequenced using ABI Prism Big Dye Terminator chemistry. Sequencing reactions were column purified and fractionated with the ABI 377 automated sequencer. DNA was sequenced from complementary strands and correction of chromatography data was facilitated by the program Sequencher 3.1.1.

Alignment and phylogenetic analysis

Alignment of histone 3 was based on conservation of amino acid reading frame. A gross alignment was performed on the ribosomal genes by manually aligning the conserved domains across all taxa in Sequencher 3.1.1. Each conserved domain, and the variable regions between the conserved domains, underwent optimization alignment using the computer program POY, run in parallel mode across an IBM SP2 Supercomputer; this is the first implementation, to our knowledge, of this program on a supercomputer. Ribosomal expansion regions were excluded from outgroups but aligned for ingroups as described elsewhere²². Sixteen parameter sets (gap/change ratio 1–4; transition/transversion ratio 1–4) were applied across all data partitions (18S rDNA, 28S rDNA, H3, and total). The parameter combination with gaps, transitions and transversions weighted equally minimized incongruence among data partitions (incongruence length difference (ILD) metric = 0.01722), and was selected as the best-justified parameter set for optimization alignment²³. Topologies from parameter combination sets adjacent to the optimal combination produced similar phylogenetic results. Optimal parameters produced a single topology (length = 9,057, consistency index = 0.508, retention index = 0.618), and the implied alignment from this topology was used to calculate bootstrap and partitioned Bremer support values. Bootstrap values were calculated in PAUP*4.0b10 (ref. 24) with 10,000 replicates, 5 random additions per replicate, and TBR branch swapping. Partitioned Bremer support values were calculated using TreeRot²⁵. The incongruence length difference test²⁶ indicated insufficient evidence to reject the hypothesis of data set congruence ($P = 0.892$), therefore individual data sets were combined into a total evidence analysis. The states 'fully winged', 'partially winged' and 'wingless' were treated as unordered characters and mapped for males and females by means of parsimony using MacClade 4.0 (ref. 27). For the ingroup, the MPR required ten steps (males) and nine steps (females), the ancestral phasmid state was reconstructed unambiguously as wingless, and wings were derived four times unambiguously. Constraining the ancestral phasmid state to fully winged (by forcing state changes as irreversible) required 28 steps for males and 30 steps for females, and is the MPR when wing gain is 11 times wing loss. Fully winged and partially winged were combined into winged and the MPR required 7 steps (males and females), the ancestral state was

reconstructed as wingless, and wings were unambiguously derived on four occasions. Mapping of characters by means of likelihood was performed using the program Discrete²⁸ on topologies generated by means of standard likelihood and bayesian methods (see Supplementary Information). Wings were mapped for males and females (character 1 and 2) with two states: wings present or absent. Character states were mapped on the maximum likelihood topology and the bayesian topology with the rate of wing loss set to a value of 1, and the rate of wing gain set to 20 individual values, spanning the interval 1 to 0.00001. Character states were also mapped on a set ($n = 30$) of bayesian trees²⁹, and the mean probability that the ancestor to Euphasmatodea was wingless is 0.9996 ± 0.000246 .

Received 29 May; accepted 31 October 2002; doi:10.1038/nature01313.

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Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com/nature>).

Acknowledgements We thank E. Tilgner for providing some specimens; A. Whiting and L. Harmon for assistance in data analysis; and J. Cherry, M. Gruwell, M. Terry, H. Ogden, J. Robertson and K. Jarvis for generating sequence data. Analyses were performed in the Fulton Supercomputer Center, Brigham Young University, and parallel software implementation was performed by M. Clement and Q. Snell. DNA sequences are deposited in GenBank under accession numbers AY121129–AY121186 and AY125216–AY125326.

Competing interests statement The authors declare that they have no competing financial interests.

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