Phylogenetic relationships of the tribe Operophterini (Lepidoptera, Geometridae): a case study of the evolution of female flightlessness

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A molecular phylogenetic analysis was conducted in order to reconstruct the evolution of female flightlessness in the geometrid tribe Operophterini (Lepidoptera, Geometridae, Larentiinae). DNA variation in four nuclear gene regions, segments D1 and D2 of 28S rRNA, *elongation factor 1α*, and *wingless*, was examined from 22 species representing seven tribes of Larentiinae and six outgroup species. Direct optimization was used to infer a phylogenetic hypothesis from the combined sequence data set. The results obtained confirmed that Operophterini (including *Malacodea*) is a monophyletic group, and Perizomini is its sister group. Within Operophterini, the genus *Malacodea* is the sister group to the genera *Operophtera* and *Epirrita*, which form a monophyletic group. This relationship is also supported by morphological data. The results suggest that female flightlessness has evolved independently twice: first in the lineage of *Malacodea* and, for the second time, in the lineage of *Operophtera* after its separation from the lineage of *Epirrita*. An alternative reconstruction (i.e. recovery of flight ability in an ancestor of *Epirrita*) appears unlikely for various reasons. The similarities shared by *Epirrita* with a basal representative of Perizomini, *Perizoma didymatum*, allow the proposal of a sequence of evolutionary events that has led to flightlessness. It is likely that the transition to female flightlessness in the two lineages of Operophterini occurred after the colonization of stable forest habitats, followed by the evolution of a specific set of permissive traits, including larval polyphagy, limited importance of adult feeding, and adult flight during the cold months of the season. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, **92**, 241–252.

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INTRODUCTION

Despite the obvious advantages of being able to fly, flight ability has secondarily been lost in various insect groups (Roff, 1990, 1994; Wagner & Liebherr, 1992). With the aim to understand costs and benefits of adult flight, several studies have been undertaken to relate the origins of flightlessness to environmental conditions. It appears that flight ability has preferentially been lost in stable habitats such as forests that provide steady resources (Barbosa, Krischik & Lance, 1989; Roff, 1990; Sattler, 1991). However, these studies have not been able to apply phylogenetic comparative methods (Harvey & Pagel, 1991) to analyse associations between flightlessness and other

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traits, primarily due to scarcity of reliable information on insect phylogenies. As an exception, Hunter (1995a) presents an attempt at a phylogeny-based analysis of female flightlessness in forest Macrolepidoptera, mainly the geometrids. However, as the author herself stressed, the phylogenetic information used was highly preliminary (i.e. not based on a rigorous cladistic analysis).

Geometrid moths, the group analysed by Hunter (1995a), may form a highly promising model taxon for studies on the evolution and ecology of female flightlessness. This is because geometrids are a large group with relatively uniform morphology and adult behaviour, but with a high, and well-documented, diversity in life histories. In some geometrid species, the flightless females are completely wingless (apterous) whereas, more typically, they are brachypterous, possessing vestigial, dysfunctional wings (Sattler, 1991). No doubt, there are several independent evolutionary transitions to female flightlessness in geometrids (Sattler, 1991; Hunter, 1995a). However, quite surprisingly, there is no contemporary phylogenetic treatment of the family, which would facilitate making full use of this favourable situation in the context of evolutionary ecology.

Most geometrid species with wingless females fit into the 'winter moth scenario' in that they share a well-defined set of ecological traits (Hackman, 1966; Barbosa *et al*., 1989). With the exception of a few species of the steppe zone, they are all forest dwellers with tree-feeding larvae. Adults of almost all of the species occur in late autumn or early spring (or throughout winter, in warmer areas), with the young larvae being able to develop on young leaves of their host trees only ('spring feeding'). As far as is known, oviposition is indiscriminatory (Tenow, 1972; Tammaru, Kaitaniemi & Ruohomäki, 1995), and eggs are often laid in clusters (a trait rare among geometrids; Sattler, 1991). The adults do not feed. Moreover, the winter moths are prone to display outbreaking population dynamics (Barbosa *et al*., 1989), with the reasons for this association not being fully understood; but see also Hunter (1995b) and Tammaru & Haukioja (1996).

The fact of the presence of evolutionary associations between the traits forming the winter moth complex may well be robust to any mistakes in the phylogenetic tree used by Hunter (1995a). In particular, there is little doubt that the different 'winter moth traits' are coadaptive, and the whole complex has arisen more than once in the course of evolution. However, detailed, and correct, phylogenetic information is needed to derive a dynamic picture of the evolution towards flightlessness. In other words, the existing knowledge does not allow us to understand the sequence of evolutionary changes that have lead to the typical winter moth scenario, including winglessness. For example, we do not know whether larval polyphagy, female flightlessness, or winter flight was the first trait of the winter moth complex to evolve.

The present study aimed to establish the phylogenetic context of transition to female flightlesness (and the winter moth scenario, in general) in the case of the geometrid tribe Operophterini. This is the only group in the large subfamily Larentiinae that contains flightless moths in the Holarctic region. Relying mainly on the methods of molecular phylogenetic analysis, the study aimed to confirm the monophyly of the tribe Operophterini and to find its closest sister group. Moreover, we investigated whether there are multiple transitions to winglessness within this tribe. Such an analysis allows us to discuss the likely ancestral states of the traits characterizing the winter moths. Above all else, however, our reconstruction represents the first step in mapping the cases of the evolution of female flightlessness on geometrid phylogeny. Along with further such cases hopefully to be resolved in future, the case of *Operophtera* will serve as raw material for phylogenetic comparative analyses of the evolution of female flightlessness.

The current subdivision of the subfamily Larentiinae into tribes dates back to the works of Herbulot (1962–1963). Most of the approximately 20 tribes form relatively uncontroversial 'natural' units, and thus the Herbulot's system has been adopted with only a few changes in recent studies (Müller, 1996; Viidalepp, 1996, 2007; Xue & Scoble, 2002; Mironov, 2003). By contrast, the phylogenetic relationships among the tribes remain largely obscure, and no formal cladistic analysis is available. A preliminary morphology-based phylogenetic tree, however, was recently proposed by Holloway (1997). At the time of writing, the only published molecular phylogeny of Geometridae was that by Abraham *et al*. (2001), which, due to the limited number of taxa included, does not form a sufficient basis for resolving the phylogenetic relationships within Larentiinae.

The tribe Operophterini, first recognized by Forbes (1948), is formed by two relatively monomorphic core genera, *Operophtera* Hübner, 1825 and *Epirrita* Hübner, 1822 (= *Oporinia* Hübner, 1925), both with wide Holarctic distribution (Inoue *et al*., 1982; Troubridge & Fitzpatrick, 1993; Viidalepp, 1996). The relatedness of these two genera has been appreciated subsequent to the study by Pierce (1914), whereas the closeness of the monotypic genus *Malacodea* Tengström, 1862 to the group (first proposed by Kuznetsov, 1929; followed by Viidalepp, 1977, 1996, 2007) has not been universally accepted (Mikkola, Jalas & Peltonen, 1985; Skou, 1986; Müller, 1996). Females of both *Operophtera* and

Malacodea are flightless whereas those of *Epirrita* bear functional wings; the internal phylogenetic structure of the tribe is thus also relevant in the context of the evolution of flightlessness.

The present phylogenetic analysis included European representatives of *Operophtera*, *Epirrita*, and *Malacodea*, along with representatives of Asthenini, Cidariini, Eupitheciini, Hydriomenini, Perizomini, and Xanthorhoini. These tribes of Larentiinae have been suggested as likely sister groups of Operopterini in earlier sources (Troubridge & Fitzpatrick, 1993; Holloway, 1997; Abraham *et al*., 2001; J. Viidalepp, unpubl. data). Using a sequence variation in four nuclear gene partitions, we present the largest molecular phylogenetic analysis performed to date of the tribes of Larentiinae focusing on the evolutionary patterns of female flightlessness in the tribe Operopterini. In some cases, it was useful to invoke morphology-based traits to provide further support for interspecies' relationships recovered by the molecular analysis.

MATERIAL AND METHODS

TAXA SAMPLED

A total of 22 species of Larentiinae representing seven tribes, Operopterini, Perizomini, Eupitheciini, Asthenini, Cidariini, Xanthorhoini, and Hydriomenini, were included in our study. Two species of Drepanidae, two geometrid species of Sterrhinae, and three species of Ennominae were used as outgroups. Drepanidae is considered to be a family closely related to geometrids, whereas Sterrhinae and Ennominae represent, along with Larentiinae, the three main subfamilies of Geometridae (Hausmann, 2001). Information on all the specimens, including the GenBank accession numbers of sequences, is given in Table 1. The sequences of 28S rRNA of seven species were provided by Abraham *et al*. (2001).

MOLECULAR TECHNIQUES

Specimens were fresh, dried, frozen or stored in ethanol. DNA was extracted from a single individual, either the entire individual or a part of it was used. DNA extraction was performed with a DNeasy Tissue Kit (Qiagen) according manufacturer's instructions. DNA was eluted in sterile water, and stored at –20 °C.

Four loci of nuclear DNA including two segments, D1 and D2 of the 28S rRNA gene, the portion of the *elongation factor 1α* (EF-1α) gene, and the portion of the *wingless* (wg) gene were studied. Polymerase chain reactions (PCR) were carried out in a total volume of 50 μL with 5–100 ng of DNA template, 0.1 μM of each primer, 200 μM of each dNTP, 1 U of DyNAzyme II DNA polymerase (Finnzymes Oy) in

$1 \times$ DyNAzyme buffer supplemented with 1.5 mM MgCl$_2$ in a PTC-100 Thermal Cycler (MJ Research Inc.). The PCR products were visualized on a 1.5% agarose gel in order to check the result of the amplification. The PCR products were purified from an aliquot of the PCR or from agarose gel band using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.). Details of PCR amplification of gene fragments are shown in Table 2.

The sequencing of the purified PCR products was performed on an automatic ABI PRISM 377 DNA Sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) following the recommended procedure. The gene fragments were sequenced in both directions (28S D1 and D2) or in one direction (EF-1α and wg) with their PCR primers.

DATA ANALYSIS

The quality of the sequences was first analysed with the sequencer's software package, Sequencher, version 3.1.1 (Gene Codes Corp.). The phylogenetic hypothesis from the combined sequence data set was inferred using direct optimization implemented by the program POY, version 3.0.11 (Wheeler, Gladstein & De Laet, 2003). POY implements heuristic searches to find the most parsimonious tree or trees with determined alignment parameter values consisting of minimum cost for transitions, transversions, and insertion-deletion (indel) events for the given data. The following POY command line was used: -fitchtrees -noleading -norandomizeoutgroup -impliedalignment -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -random 5 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -numdriftchanges 30 -driftspr -numdriftspr 10 -drifttbr -numdrifttbr 10 -slop 10 -checkslop 10.

D2 sequences were highly length variable and, when used as such in a direct optimization, the results were ambiguous. Therefore, the D2 sequences were cut into four fragments, each of which started and ended with conserved sequence block. Ten different alignment cost parameter values were explored: 1 : 1 : 1 (transiton : transversion : indel), 1 : 1 : 2, 1 : 1 : 4, 1 : 1 : 6, 1 : 1 : 8, 1 : 2 : 1, 1 : 2 : 2, 1 : 2 : 4, 1 : 2 : 6, and 1 : 2 : 8. D1, and EF-1α showed no length variation and were unambiguously aligned. The wg sequences of all Larentiinae species showed two codon insertions, which were between positions 115/116 and 127/128 in the *Colobura dirce* Linnaeus, 1758 wg sequence (GenBank accession number AY090162). The latter three partitions were treated as prealigned during direct optimization. The

Table 1. Information on the specimens used in this study

Species	Collection site and year	Collector	28S D1	28S D2	EF-1α	wingless
Geometridae						
Larentiinae						
Operophterini						
Epirrita autumnata (Borkhausen, 1794) larva	FIN, Masku, 2000	K. Ruohomäki	AY948443	AY948463	AY948485	AY948513
Epirrita christyi (Allen, 1906)	FIN, Seili, 2000	K. Ruohomäki	AY948444	AY948465	AY948486	AY948514
Epirrita dilutata (Denis & Schiffermüller, 1775)	FIN, Husä, 2001	M. Östman	AY948445	AY948466	AY948487	AY948515
Operophtera brumata (Linnaeus, 1758), larva	FIN, Turku, 2000	K. Lempa	AF178891*	AF178915*	AY948488	AY948516
Operophtera fagata (Scharfenberg, 1805)	FIN, Pori, 1997	K. Ruohomäki	AF178892*	AF178916*	AY948489	AY948517
Malacodea regelaria (Tengström, 1869)	EST, Puka, 1994	J. Viidalepp	AY948446	AY948467	AY948490	AY948518
Perizomini						
Perizoma albulatum (Denis & Schiffermüller, 1775), larva	FIN, Uusikaupunki, 2001	S. Puustinen	AY948447	AY948468	AY948491	AY948519
Perizoma didymatum (Linnaeus, 1758)	FIN, Seili, 2001	K. Ruohomäki	AY948448	AY948469	AY948492	AY948520
Perizoma alchemillatum (Linnaeus, 1758)	FIN, Seili, 2001	K. Ruohomäki	AY948449	AY948470	AY948493	AY948521
Eupitheciini						
Eupithecia innotata (Hufnagel, 1767)	FIN, Reposaari, 2001	K. Ruohomäki	AY948450	AY948471	AY948494	AY948522
Eupithecia icterata (Villers, 1789)	EST, Elva, 1999	T. Tammaru	AY948451	AY948472	AY948495	AY948523
Astheniini						
Discoloxia blomeri (Curtis, 1832)	EST, Elva, 1999	T. Tammaru	AY948452	AY948473	AY948496	AY948524
Euchoeca nebulata (Scopoli, 1763)	EST, Tartu, 1997	T. Tammaru	AY948453	AY948474	AY948497	AY948525
Hydrelia flammeolaria (Hufnagel, 1767)	EST, Tartu, 1997	T. Tammaru	AY948454	AY948475	AY948498	AY948526
Cidariini						
Thera firmata (Hübner, 1822)	EST, Kauksi, 1999	T. Tammaru	AY948455	AY948476	AY948499	AY948527
Eulithis populata (Linnaeus, 1758)	FIN, Seili, 2001	K. Ruohomäki	AY948456	AY948477	AY948500	AY948528
Eulithis testata (Linnaeus, 1761)	FIN, Seili, 2001	K. Ruohomäki	AY948457	AY948478	AY948501	AY948529
Chloroclysta citrata (Linnaeus, 1761)	FIN, Seili, 2001	K. Ruohomäki	AY948458	AY948479	AY948502	AY948530
Ecliptopera silaceata (Denis & Schiffermüller, 1775)	EST, Avinurme, 2004	T. Tammaru	AF178893*	AF178917*	AY948503	–
Xanthorhoini						
Scotopteryx chenopodiata (Linnaeus, 1758)	FIN, Seili, 2001	K. Ruohomäki	AY948459	AY948480	AY948504	AY948531
Epirrhoe alternata (Müller, 1764)	FIN, Seili, 2001	K. Ruohomäki	AY948460	AY948481	AY948505	AY948532
Hydriomenini						
Hydriomena furcata (Thunberg, 1784)	FIN, Parainen, 1997	K. Ruohomäki	AF178895*	AF178919*	AY948506	AY948533
Sterrhinae						
Idaea straminata (Borkhausen, 1794)	EST, Kauksi, 1998	T. Tammaru	AF178889*	AF178913*	AY948507	AY948534
Scopula immorata (Linnaeus, 1758)	EST, Karilatsi, 2002	T. Tammaru	AF178886*	AF178910*	AY948508	AY948535
Ennominae						
Lycia hirtaria (Clerck, 1759)	EST, Avinurme, 1999	T. Tammaru	AY948461	AY948482	AY948509	AY948536
Apocheima pilosaria (Denis & Schiffermüller, 1775)	EST, Avinurme, 1999	T. Tammaru	AY948462	AY948483	AY948510	AY948537
Paleacrita merriccata (Dyar, 1903)	USA, MD, 2000	T. Tammaru	AY948463	AY948484	AY948511	AY948538
Drepanidae						
Drepana lacertinaria (Linnaeus)	–	–	AF178882*	AF178906*	–	–
Drepana curvatula (Borkhausen, 1790)	FIN, Hanko, 2002	K. Ruohomäki	–	–	AY948512	AY948539

Three larvae were used, the rest of the specimens were adult. Collection site (FIN, Finland; EST, Estonia) and year, collector's name, and GenBank accession numbers of sequences are indicated.

*Abraham *et al.* (2001); –, not available or not used.

Table 2. Details of polymerase chain reaction primers and amplification conditions of gene fragments 28S D1 and D2, EF-1α and wgl

Gene fragment	Primer pairs	Ref for primer sequences	PCR conditions
28S D1	D1 For: 5′-GGG GAG GAA AAG AAA CTA AC-3′ D1 Rev: 5′-CAA CTT TCC CTT ACG GTA CT-3′	Larsen (1992)	95 °C/5 min; 95 °C/30 s, 51 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/5 min
28S D2	D2 For: 5′-AGA GAG AGT TCA AGA GTA CGT G-3′ D2 Rev: 5′-TTG GTC CGT GTT TCA AGA CGG G-3′	Belshaw & Quicke (1997)	95 °C/5 min; 95 °C/30 s, 54–55 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/5 min
EF-1α	EF44: 5′-GCY GAR CGY GAR CGT GGT ATY AC-3′ EFrcM4: 5′-ACA GCV ACK GTY TGY CTC ATR TC-3′	Monteiro & Pierce (2001)	95 °C/7 min; 95 °C/1 min, 55–56 °C/45 s–1 min, 72 °C/1–2 min, 35 cycles; 72 °C/10 min
5′ half of EF-1α	Cho2: 5′-CTA CGT CAC CAT CAT CGA-3′ Verdi4: 5′-CAC CAG TCT CCA CAC GGC C-3′	Present study	95 °C/7 min; 95 °C/30 s, 57.5 °C/30 s–1 min, 72 °C/1 min, 35 cycles; 72 °C/10 min
3′ half of EF-1α	EF51.9: 5′-CAR GAC GTA TAC AAA ATC-3′ EFrcM4	Monteiro & Pierce (2001)	95 °C/7 min; 95 °C/30 s, 55 °C/1 min, 72 °C/1 min, 35 cycles; 72 °C/10 min
3′ half of EF-1α	Niina: 5′-CCA CAG ACA AGG CCC TGC G-3′ Niina2: 5′-CCT GGA AGG ACT CCA CRC ACA G-3′	Present study	95 °C/7 min; 95 °C/40 s, 55–56 °C/1–1.30 min, 72 °C/1 min, 35 cycles; 72 °C/10 min
wgl	LepWG1: 5′-GAR TGY AAR TGY CAY GGY ATG TCT GG-3′ LepWG3: 5′-ACT YCG CAR CAC CAR TGG AAT GTR CA-3′	Brower & DeSalle (1998)	95 °C/5 min; 95 °C/30 s, 49–50 °C/1 min, 72 °C/1 min, 35 cycles; 72 °C/10 min

The upper primer of the primer pair is forward primer and the lower one is reverse primer. The EF-1α fragment was amplified either as whole or in two pieces, which partly overlapped. For the amplification of the 3′ part of the EF-1α fragment, there are two possible primer combinations.

combinability of the gene partitions was tested by using the incongruence length difference test (Farris *et al*., 1994).

Choosing between the different resulting phylogenetic hypotheses generated by the direct optimization analyses proved to be difficult and subjective. Thus, the elision procedure (Wheeler, Gatesy & DeSalle, 1995) was used. One implied alignment of each D2 segment from each of the POY analyses with different parameters were concatenated into a single grand matrix. All characters in this matrix were downweighted by a factor of 10 (i.e. the number of parameter combinations used) and the matrix was analysed with the other gene regions (which kept their weight of one). This has the effect of giving a weight of one to character sets in the D2 partition that are recovered in all POY runs, down to a weight of 0.1 to character sets found in only one of the POY runs (Wheeler *et al*., 1995).

The differentially weighted concatenated datasets were analysed with WinClada, version 10.00.08 (Nixon, 2002), running NONA, version 2.0 (Goloboff, 1999) to find the most parsimonious tree(s). Support for branches was analysed using bootstrap and Bremer support. Bootstrapping was performed with one thousand pseudoreplicates, each with five search replications and two starting trees per replication. Bremer support values (Bremer, 1988) were calculated using the program PAUP 4.0 (Swofford, 2001) in conjunction with TreeRot (Sorensen, 1999). Both support measures were taken from the elized data set, resulting in Bremer support values that are not whole numbers.

RESULTS

SEQUENCE LENGTH VARIATION

Of the four nuclear gene fragments studied, the D1 and D2 fragments of 28S rRNA gene were successfully sequenced for all taxa, but the PCR reactions of the wg and the 5′ part of the EF-1α failed for one species, *Ecliptopera silaceata* and *Scopula immorata*, respectively. The length of the D1, 295 bp, was constant for all taxa studied. The length of D2 and wg varied due to indels, with D2 varying between 424 and 474 bp, and wg varying between 404 and 398 bp. For EF-1α, 1020 bp were sequenced, although this was less for the specimens in which EF-1α was amplified in two pieces.

The outgroup of the Drepanidae comprised two species, *Drepana curvatula* and *Drepana lacertinaria*. The D2 sequence of *D. curvatula* showed anomalous regions, which confounded alignment attempts, whereas the D2 of *D. lacertinaria* was more conservative. On the other hand, PCR reactions for EF-1α

and wg were not successful in *D. lacertinaria*, whereas they were for *D. curvatula*. Therefore, the sequences of the two drepanid species were combined so that, for the phylogenetic analysis, the D1 and D2 sequences were obtained from *D. lacertinaria* and EF-1α and wg sequences from *D. curvatula*.

PHYLOGENETIC ANALYSIS

Direct optimization of the molecular data using a variety of parameter values resulted in several stable relationships (shown as bold branches in Fig. 1). The subfamily Larentiinae was found to be monophyletic under all combinations of parameter values that were investigated. At the tribal level, Operophterini, Perizomini, Eupitheciini, and Asthenini were also stable monophyletic groups, as was the group of the three tribes Hydriomenini + Xanthorhoini + Cidariini. The position of Perizomini as the sister group to Operophterini was stable to all parameter value combinations, as was the sister group relationship of Hydriomenini + Xanthorhoini + Cidariini and Operophterini + Perizomini.

Maximum parsimony analysis of elized data matrix resulted in one most parsimonious tree (Fig. 1), which was identical to the tree recovered in the POY analyses with ts : tv : gap costs of 1 : 1 : 2 and 1 : 1 : 8, as well as one of the two trees recovered in the analyses with parameters 1 : 1 : 1, 1 : 1 : 4 and 1 : 1 : 6. Support values based on the elized dataset showed that the branches that are stable to various parameter combinations are also well supported (bootstrap generally > 70%, Bremer support generally > 5).

Traditional tribes within Larentiinae were generally well supported, with exception of the tribe Cidariini. Within the clade Cidariini + Xanthorhoini + Hydriomenini, the elized data did not place Cidariini as monophyletic group as *Ecliptopera silaceata* grouped with the Hydriomenini (the species *Hydriomena furcata*). Thus, this placement receives low support and is not stable. Instead, our results are consistent with the idea that Xanthorhoini is monophyletic. In the phylogenetic hypothesis presented (Fig. 1), the clade formed by Eupitheciini was placed as the sister to the rest of Larentiinae sampled in this study. However, this finding did not receive much support and was not stable because it was not recovered in every analysis.

The molecular analysis showed that Operophterini and Perizomini formed a monophyletic clade, in which they are placed as sister groups. Within the monophyletic Perizomini, the relationships were very well resolved, *Perizoma didymatum* being placed sister to *Perizoma alchemillatum* and *Perizoma albulatum*. The monophyly of Operophterini (including *Malacodea*), recovered with strong support, is also sup-
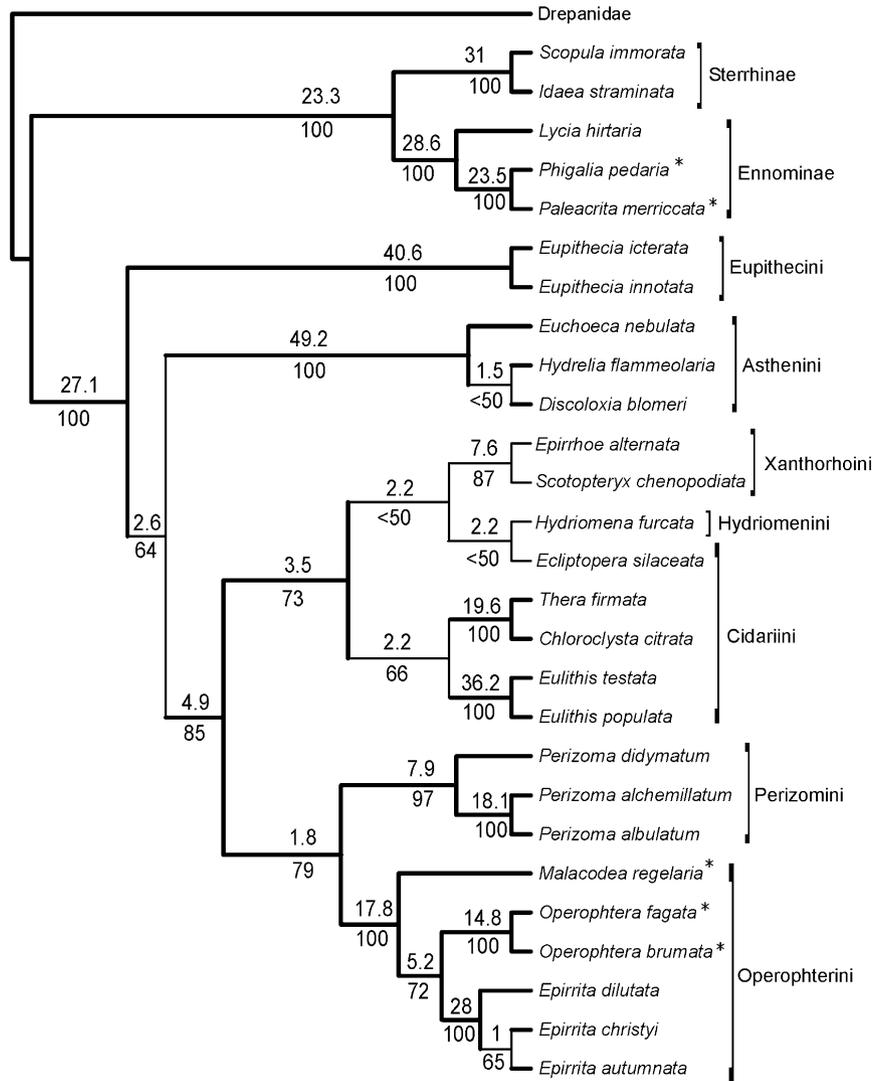
Figure 1. The most parsimonious tree from the combined data set of four nuclear gene partitions (using the elision method; see text for details). Numbers above branches are Bremer support values and numbers below branches are bootstrap values. Thick branches are stable of the transition:transversion:gap cost parameter values used during direct optimization. Species with flightless females are indicated by an asterisk.

ported by the morphological evidence based on the five synapomorphies characterizing the tribe (Table 3). Within Operophterini, although the phylogenetic relationships were supported variably, branching structure and order in the most parsimonious tree were stable, except for the genus *Epirrita*, where the placement of *Epirrita dilutata* basal to *Epirrita christyi* and *Epirrita autumnata* is uncertain. *Malacodea* was revealed, with moderate node support, as the most basal branch of the tribe, so that the genera *Operophtera* and *Epirrita* formed a monophyletic group. Such a relationship was also strongly supported by morphological data. In particular, *Operophtera* and *Epirrita* share three morphological

synapomorphies (one of which is a pupal character: apical setae in cremaster) (Table 4), absent in *Malacodea*. The presented phylogenetic structure (Fig. 1) is thus supported by several lines of evidence. Due to potential logical circularity related to the use of the gene *wingless* (known to be essential in insect wing formation) in an analysis of winglessness, the analysis was repeated with the data with wg gene excluded. Such an alternative analysis did not lead to any different results with respect to the most likely sistergroup of Operophterini, and the internal structure of the tribe. Furthermore, the incongruence test showed that wg is not incongruent ($P = 0.2673$) with the other combined data sets.

Table 3. Synapomorphies characteristic of Operophterini (Viidalepp, 2007)

Synapomorphy	Operophterini	By contrast to:
Male genital armature	With transtilla band-shaped and sclerotized	Not sclerotized
Frenulum in male	Reduced or nonfunctional	Present and functional
Abdominal tympanal cavities	Smaller in females than males	Not sexually different
Clypeus naked	With a projecting lateroventral teeth	Not dentate lateroventrally
Extra sclerite lateral to clypeus	Present	Absent

Table 4. Morphological synapomorphies of *Epirrita + Operophtera* versus *Malacodea* (Viidalepp, 2007)

Synapomorphy	*Epirrita + Operophtera*	Malacodea
Apical setae in cremaster	Fused T- or Y-shaped	Hooked
Anal papillae in female	Conical	Rounded
Genital apophyses in female	Stronger, flattened	Weaker, filiform

DISCUSSION

PHYLOGENETIC RELATIONSHIPS WITHIN LARENTIINAE

Based on the current nuclear DNA data, the studied members of Larentiinae appeared to form a monophyletic group. The representatives of the geometrid subfamilies of Ennominae and Sterrhinae grouped together and appeared as the sistergroup to Larentiinae, which remains monophyletic also when the number of Ennominae species is considerably increased (N. Snäll, unpubl. data).

Within Larentiinae, the results were consistent with the monophyly of the tribes Operophterini, Perizomini, Eupitheciini, Asthenini, and Xanthorhoini whereas our results suggest that the tribe Cidariini might be polyphyletic because one of its members, *Ecliptopera silaceata* (representing a basal branch of the tribe, as suggested by Choi, 1997), grouped together with *Hydriomena furcata*, the only representative of the tribe Hydriomenini in this study. The evidence for the polyphyly of Cidariini is weak, however, because all nodes separating *E. silaceata* from the rest of Cidariini received very low support and, moreover, *E. silaceata* was the only species in which the PCR amplification of the wg gene failed. Thus, it is well possible that the missing data may have affected the result.

The monophyly of Operophterini (including *Malacodea*) was clearly confirmed. The appearance of this tribe in the cladogram as one entity was strongly supported and invariably stable regardless of the parameter values. Perizomini was shown to be the sister group to Operophterini, supporting the early anticipation by Pierce (1914). However, the relatedness of Perizomini and Operophterini has not been universally accepted (Viidalepp, 1996), with the main reason being the absence of well-defined morphological synapomorphies for the clade Perizomini + Operophterini. One morphological synapomorphy of these tribes can still be proposed: the *labides* (a structure in male external genitalia) are fused above the *aedeagus* in both Perizomini and Operophterini (J. Viidalepp, unpubl. data).

A MULTIPLE ORIGINS OF FEMALE WINGLESSNESS IS LIKELY

Females of the monotypic genus *Malacodea*, as well as those of all *Operophtera* species, are flightless (Inoue *et al*., 1982; Troubridge & Fitzpatrick, 1993) whereas the females of *Epirrita* bear functional wings. The phylogenetic hypothesis proposed here for Operophterini (Fig. 1) thus allows two alternative interpretations regarding the evolution of female flightlessness. First, the loss of female wings may have occurred in a common ancestor of all recent Operophterini, implying that female flight would have re-evolved in *Epirrita*. Second, female flightlessness might have evolved twice: one transition occurred in an ancestor of *Malacodea*, another after the separation of *Operophtera* from *Epirrita*. These two scenarios are equally parsimonious if equal weights are assigned to the evolution of flightlessness, and reversal to the volant phenotype. However, even if the first scenario (i.e. a reversal to the winged phenotype in *Epirrita* cannot perhaps be totally excluded), we are strongly inclined to consider the

second scenario considerably more likely for reasons discussed below.

First, re-evolving wings, as well as any other complex morphological structure lost in the course of evolution has traditionally been considered improbable, or even impossible (Porter & Crandall, 2003; West-Eberhard, 2003). In the line with this premise, it has been a long-held notion in evolutionary entomology that wings evolved just once and have subsequently been lost in a number of lineages. Recently, however, this view was challenged by Whiting, Bradler & Maxwell (2003) who reconstructed winglessness as the ancestral state for the order Phasmatodea. This allowed them to conclude that recovery of flight ability had occurred as many as four times during the evolution of stick insects. Such a conclusion was, nevertheless, not received with unanimous approval (Trueman *et al.*, 2004). It thus still holds that there is no single universally accepted case of re-evolved flight ability among insects whereas there is a very high number of indisputable cases of wing loss (Roff, 1990). For the time being at least, it appears to be a sensible option to consider the loss of wings as a much more likely evolutionary event than wing recovery.

However, the typical case of moths still differs from many others in one important aspect: wing reduction affects only one sex. This implies that the functionality of the set of genes responsible for the development of functional wings is continuously maintained by natural selection, and restoring wings in females, might, in principle be the question of a mutation in a single regulatory gene. Nevertheless, the adaptive behaviour of a winged female is a trait not displayed in males. It is thus perhaps reasonable to assume that a female would not be able to make proper use of her suddenly re-evolved wings, after millions of years of brachypterous evolutionary history. Most likely, such a female would not be able to deal adaptively with her considerably increased apparency to predators, and the re-evolved winged phenotype would be strongly selected against.

Most importantly, however, in Lepidoptera, the absence of wings is not the only, nor even the main feature of brachypterous phenotypes. Brachyptery affects much more than the wings (Sattler, 1991): typically, profound changes in the composition of the whole body have occurred in connection to the evolution of flightlessness. Such females, including those of flightless Operophterini, have notably large abdomens but a strongly reduced thorax and head, so the absence of wings per se is not the only trait causing their inability to fly. This is best illustrated by the well-known example of some populations of gypsy moth (*Lymantria dispar* Linnaeus): the heavy-bodied females of this species are unable to fly despite of possessing full-sized wings (Leonard, 1981).

Even if female wings could be restored as a consequence of a few mutations, proportions of the parts of adult body most likely represent a polygenic, gradually and slowly evolving trait. This is because various intermediate states can be found among Lepidoptera, including geometrids, and the trait appears to be conservative phylogenetically: the property of having heavy, flight-impeding female abdomens is fixed at the genus level (or above) among at least European Macrolepidoptera. Moreover, by contrast to the frequent within-species polyphenism with respect to possessing wings in various insects (Zera & Denno, 1997), we are unaware of any documented cases of considerable phenotypic plasticity with respect to allocating resources to abdomens (fecundity) versus thorax (mobility) in Lepidoptera. Additionally, in insects, the phenotypic effects of single mutations on body composition (best known for *Drosophila*; Lindsley & Grell, 1968) are truly limited in comparison to the differences between actively flying and wingless species of geometrids. It is also relevant in this context that mutations leading to the absence of wings (the respective gene network is reasonably well understood, and relatively invariable among insects; Carroll *et al.*, 1994; Abouheif & Wray, 2002) appear to have no considerable pleiotropic effects on body composition either in *Drosophila* (Lindsley & Grell, 1968), nor in *Bombyx* (Goldsmith & Wilkins, 1995), the lepidopteran best studied genetically.

An evolutionary change from a heavy-bodied wingless female phenotype back to an actively flying one should thus be seen as a gradual rather than an abrupt one. Critically, however, it is hard to determine which selective forces could have favoured a gradual decrease in abdomen size before full flight ability is restored. Nothing appears to be able to compensate for the associated reduction in potential fecundity before the benefits of increased mobility can be enjoyed.

In this context, the lack of adult feeding capability, a trait associated with the brachypterous phenotype, should also be discussed. Neither sex of any of the (North European, at least) flightless lepidopterans feed as adults, whereas most other moths do. Adults of *Epirrita* do feed (Tammaru, Kaitaniemi & Ruohomäki, 1996), whereas those of *Operophtera* do not. If the common ancestor of *Operophtera* and *Epirrita* was a typical female-wingless geometrid, *Epirrita* would most likely have to re-evolve not only female wings, but also adult feeding capability. This might have been even more complicated because both sexes were probably lacking a functional digestive system. In summary, various lines of evidence appear to support the hypothesis of a multiple origins of flightlessness in Operophterini, rather than that of wing recurrence in *Epirrita*.

EVOLUTIONARY ECOLOGY OF FLIGHTLESSNESS: A HYPOTHESIS

As far as known, *Malacodea* and *Operophtera* are the only genera with brachypterous females among Holarctic Larentiinae (Sattler, 1991), a large group including several hundreds of species. The assumption of two independent transitions occurring in two closely-related lineages, and not anywhere else throughout the high diversity of the subfamily, strongly suggests that the clade was predisposed to such an evolutionary event taking place. This implies that female flightlessness was not the first trait of the winter moth complex to evolve. This event was preceded by at least some evolutionary changes making the transition to flightlessnes likely.

To be able to put forward candidates for such permissive traits, we would need to reconstruct the states of life history traits in the ancestor of Operophterini. Here, the sister group comes into use. We can note that *Epirrita*, the only winged representatives of Operophterini, bear remarkable similarities with the basal representative of Perizomini, *Perizoma didymatum*. Both species are polyphagous forest dwellers, with the egg as the overwintering stage. The overwintering stage also determines the relatively late flight period (early autumn in *Epirrita*, late summer in *P. didymatum*). Notably, both deviate from the typical larentiine moths showing a moderate reduction in female wing size (i.e. wings of females smaller than those of males), and heavy abdomens in females. These traits are observable also in some other representatives of Perizomini, such as *Perizoma verberata* Scopoli, 1763 (Mironov, 2003). Moths with such phenotype are known as poor fliers shown for the ecologically well-studied *Epirrita* (Itämies, Pulliainen & Siekkinen, 1995; Tammaru *et al.*, 1995; Ruohomäki *et al.*, 2000).

It is reasonable to assume that the traits which *Epirrita* and the basal Perizomini have in common might have been characteristic also of the ancestor of modern Operophterini. Furthermore, *P. didymatum* and *Epirrita* spp. may be seen as stepping stones towards the evolution of female flightlessness. Making such an assumption, the following hypothetical evolutionary scenario appears to be likely. A remote ancestor of Operophterini was a typical larentiine moth, living in open landscape, and using various herbs as larval hosts. Then it colonized forests, and included deciduous trees in the larval diet (as is the case in *P. didymatum*). As the nutritional quality of foliage of deciduous trees is much higher early in the season (Ayres & MacLean, 1987; Hunter & Lechowicz, 1992), larval feeding period shifted to spring, so that the egg became the overwintering stage, and adults were flying in late summer. Female mobility lost its crucial significance for at least four complementary reasons: the forest habitat is stable in general (Roff, 1990), suitable host plants are abundant in forests (Barbosa *et al.*, 1989), selecting individual host plants according to foliage quality is not effective late in the season, and the sources of adult food are scarce in late summer.

It became beneficial to invest in heavy female abdomens (i.e. majority of eggs fully developed at adult eclosion, resulting on higher fecundity and fast oviposition) instead of flight performance (i.e. light body composition required). Once the females eclose with egg loads sufficient to reduce their flight performance, it becomes increasingly less favourable to rely on adult foraging for reproduction. In turn, the less importance given to adult foraging, the more advantageous large egg loads become, the circle closes, and the evolutionary path towards capital breeding (relying on larval-derived resources in reproduction, instead of adult foraging, Tammaru & Haukioja, 1996) becomes open. As soon as female behaviour loses its crucial role, it becomes advantageous to shift the adult period to late autumn to reduce the risk of predation (Tammaru *et al.*, 2001; Tanhuanpää, Ruohomäki & Kaitaniemi, 2003), and to lose wings to increase crypsis (Sattler, 1991), and perhaps also to avoid the risk of being blown out of the habitat. Being a polyphagous forest moth may thus be seen as a permissive trait for flightlessness to evolve. Naturally, we fully acknowledge that the above construction is nothing more than a hypothesis, or an 'adaptive story'. Elements of this hypothetical scenario can, however, be rigorously tested when other cases of the evolution of flightlessness become resolved phylogenetically.

As an aside, it is interesting to note that another lineage within the Perizomini, represented in our analysis by *P. albulatum* and *P. alchemillatum*, has evolved in a contrasting direction, and differs diametrically from the winter moths. Likely, the key feature was specialization to feed on plant seeds, which has brought about monophagy, mobility, small size, and midsummer flight as covarying traits, all with intuitive adaptive explanations.

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