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The evolution of female flightlessness among Ennominae of the Holarctic forest zone (Lepidoptera, Geometridae)

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ABSTRACT

In order to facilitate the study of the evolution of female flightlessness among the geometrid subfamily Ennominae (Lepidoptera, Geometridae), we carried out a phylogenetic analysis based on a morphological data matrix, and DNA sequences. We used seven nuclear gene fragments, *elongation factor 1 α* (EF-1 α), *wingless* (*wgl*), *isocitrate dehydrogenase* (IDH), *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), *ribosomal protein S5* (RpS5) and segments D1 and D2 of the 28S rRNA gene, and one mitochondrial gene fragment, *cytochrome oxidase subunit I* (COI). Sampling included 55 species of Ennominae covering all tribes with flightless females of the Holarctic boreal zone, and some other geometrids used as outgroups. Our results clearly confirmed that Ennominae (including *Alsophila* of the traditional subfamily Alsophilinae) is a monophyletic group, as well as supported the previously established morphology-based division of Ennominae into “ennomine” and “boarmiine” groups of genera. A number of taxonomic ambiguities were resolved but the monophyly of the traditionally recognised tribe Bistonini, comprising a number of flightless species, remained ambiguous. Bistonini is thus suggested to be subsumed to the tribe Boarmiini in the broad sense. Indeed, an analysis of timing of divergence suggested that Boarmiini s. lat. rapidly diversified in the late Oligocene/early Miocene. Within the Ennominae, seven independent origins of female flightlessness were revealed facilitating phylogenetic comparative analyses to be performed in search of causes and consequences of this phenomenon. The present phylogenetic hypothesis supports the conclusions of the “adaptive story”, a hypothesis of the sequence of evolutionary events leading to flightlessness, we have presented earlier (Snäll et al., 2007). In particular, in the “boarmiine” group, the tribe Boarmiini s. lat. clearly represents a group of geometrids in which female flightlessness has evolved more frequently than in any other tribes, suggesting that this clade has likely been predisposed to evolutionary events leading to the manifestation of female flightlessness. The ancestor of the wing-reduced Ennominae has likely been a winged but slow flying forest moth feeding polyphagously on deciduous trees.

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1. Introduction

It is believed that the principal factor enabling the huge evolutionary success of insects has been the ability to fly. Nevertheless, this ability has been secondarily lost in various insect groups (Byers, 1969; Roff, 1990; Wagner and Lieberr, 1992) implying that flightlessness should offer certain benefits as well. Indeed, it has been shown in some systems that being flightless can provide an insect with notable fitness advantages, e.g. greater fecundity and an earlier start to reproduction (Wagner and Lieberr, 1992). An experimentally based analysis of possessing wings in the conceptual framework of evolutionary ecology is complicated by the

fact that the coexistence of flying and flightless morphs of a species is largely limited to particular taxonomic and ecological groups. Inferences concerning evolutionary causes and consequences of flightlessness should therefore preferably be based on phylogenetic comparative analyses (Harvey and Pagel, 1991), and a reliable phylogenetic reconstruction of the target insect group is thus crucial.

Being one of the five lepidopteran families which also include species with reduced wings (Sattler, 1991), the species-rich Geometridae – along with Psychidae (Rhainds et al., 2008, 2009) – constitute a highly suitable taxon for studying origins and consequences of flight loss. Indeed, Hunter's (1995) ecologically oriented survey of female flightlessness among forest-inhabiting Macrolepidoptera largely relied on geometrids, and the case has become a textbook example illustrating various aspects of the

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evolutionary ecology of forest insects (Price, 1997; Speight et al., 2005). This example, however, suffers from clearly deficient phylogenetic information. In fact, Geometridae still lack a phylogenetic hypothesis large and detailed enough to provide an adequate basis for analyses concerning associations between flightlessness and other traits. Inevitably, therefore, the two methodologically up-to-date studies which either directly (Snäll et al., 2007) or indirectly (Yamamoto and Sota, 2007) trace the evolution of female flightlessness in Geometridae could only have a very narrow taxonomic focus.

Within Geometridae, wing reduction is restricted to the female sex (Roff, 1990; Sattler, 1991; Hunter, 1995). Most flightless geometrid females are brachypterous, but species with completely apterous females also exist (Sattler, 1991). The adults of most known geometrids with flightless females are northern hemisphere cold season species forming the so-called ecological group of winter moths. The “winter moth syndrome” is a set of ecological traits which includes adult flight season either very early or very late in the season (or the moths are flying throughout the winter in warmer areas), preferring forest habitats, clustering of eggs (a trait rare among geometrids), overwintering as eggs or as pharate adults, larval polyphagy, spring-feeding larvae, no or limited adult feeding, and a tendency for outbreeding population dynamics (Hackman, 1966; Barbosa et al., 1989; Tammaru et al., 2001).

Wing reduction occurs in three traditionally recognized geometrid subfamilies: Alsophilinae, Larentiinae and Ennominae (Sattler, 1991). Of these, Ennominae is the largest, consisting of around 9700 described species worldwide placed in approximately 1100 genera (Minet and Scoble, 1999). Within Ennominae, species can be divided into the “ennomine” and “boarmiine” groups based on the structure of the cremaster in the pupal stage (Forbes, 1948; Holloway, 1993; Patočka and Turčani, 2005; Viidalepp et al., 2007). Beyond this major subdivision, the phylogenetic relationships among the numerous traditionally recognised tribes of Ennominae have remained largely uncertain (see Holloway, 1997 for a recent morphology-based hypothesis). In particular, the status and exact delimitation of the tribe Bistonini has remained controversial, with Holloway (1993) treating this taxon as a subgroup of his broadly defined Boarmiini. However, the status of Bistonini, and particularly the question of monophyly of this group is of primary importance in the present context as the tribe includes most geometrid species with flightless females, at least in the Holarctic region.

Molecular-based studies concerning the phylogeny of geometrids are still sparse. The first molecular phylogeny of Geometridae was published as recently as 2001 (Abraham et al., 2001). To date, there are just two larger scale studies of molecular relationships: the one by Young (2006), being based on a sample of Tasmanian species mainly; and an analysis of a limited set of Japanese taxa (Yamamoto and Sota, 2007). There are, however, an increasing number of more focussed taxon specific molecular studies on geometrids (e.g. Snäll et al., 2007; Viidalepp et al., 2007; Öunap et al., 2008; Öunap and Viidalepp, 2009).

The goal of the present study was to investigate the phylogenetic relationships within the geometrid subfamily of Ennominae with the primary aim to trace the number of independent evolutionary lineages of female flightlessness. This information is seen as a necessary precondition for subsequent phylogenetically based analyses of female flightlessness, and its ecological correlates. To resolve this question, we obtained original nucleotide sequences of 40 Ennominae of the Holarctic forest zone. The combined nucleotide data comprises seven nuclear gene fragments, *elongation factor 1 α* (EF-1 α), *wingless* (wgl), *isocitrate dehydrogenase* (IDH), *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), *ribosomal protein S5* (RpS5) and segments D1 and D2 of 28S rRNA gene, and one mitochondrial gene fragment, *cytochrome oxidase subunit I* (COI).

DNA sequence data of EF-1 α , wgl and COI have frequently been utilized in many phylogenetic studies of Lepidoptera (Monteiro and Pierce, 2001; Wahlberg and Nylin, 2003; Mallarino et al., 2005; Silva-Brandão et al., 2005; Wahlberg et al., 2005; Young, 2006; Yamamoto and Sota, 2007), whereas 28S has mainly been used in studies of geometrid moths (Abraham et al., 2001; Young, 2006; Snäll et al., 2007; Yamamoto and Sota, 2007). Although the use of mtDNA genes has traditionally been abundant, nuclear genes have been shown to provide better resolution for analyses of higher taxonomic levels (Brower and DeSalle, 1998; Caterino et al., 2000; Nazari et al., 2007; Wahlberg and Wheat, 2008). In this study, we increase the number of nuclear genes to include some of the new gene regions reported by Wahlberg and Wheat (2008) and explore their use in geometrid moths. The combined analyses of nuclear and/or mitochondrial data sets tend to provide a better supported phylogenetic hypothesis than a single marker alone (Abraham et al., 2001; Niehuis et al., 2006; Wahlberg and Wheat, 2008).

Moreover, to resolve the phylogenetic relationships in the crucial, and simultaneously the most complicated group, the Boarmiini in the broad sense, sequences of EF-1 α and 28S were retrieved from GenBank for six Japanese species, as based on Yamamoto and Sota (2007). Furthermore, in addition to the DNA sequence data set, a matrix of morphological traits was compiled which facilitated a combined analysis of morphological and genetic data. As a result, we were able to trace independent origins of female flightlessness in the subfamily Ennominae, and to contribute to solving various taxonomical problems simultaneously. The results are discussed in the framework of evolutionary ecology of female flightlessness.

2. Materials and methods

2.1. Sampling of taxa

DNA sequences of a total of 49 species of Ennominae and six outgroups from the Holarctic forest zone were examined (Table 1). Sampling covered most tribes of this geometrid subfamily represented in northern Europe, including all genera with flightless females. Our bias towards the north European fauna is justified by our intention to use the present study as a basis for comparative analyses of traits putatively correlated with female flightlessness. Collecting data to be used in such analysis requires rearing the species in the laboratory which is primarily feasible for species occurring in the study area of the researchers. Nevertheless, adding a few species from the Nearctic region, as well as the possibility to use published sequences of a number of Japanese species allowed our analysis to cover most genera with flightless geometrids from the entire Holarctic region.

Species from other geometrid subfamilies, three species of Larentiinae, two species of Sterrhinae, two species of Geometrinae, one species of Archiearinae and one species of Alsophilinae, were also included. Two species of Drepanidae were used as outgroups. Taxon names, and collection information, together with GenBank accession numbers are listed in Table 1.

For a morphological study on Boarmiini s. lato, >70 species were studied as based on availability of collection material (Viidalepp et al., 2007). In the final stage, however, the morphologically studied sample of species was pruned to overlap with the one used in molecular analyses, with a specific emphasis on Boarmiinae s. lat. In one occasion (*Milionia*), different closely related congeners were available for genetic and morphological studies. In this case, the respective genus is represented by combined data from two sibling species in the joint phylogenetic analysis of molecular and morphological data.

Table 1
Information on specimens used (EST – Estonia, FIN – Finland, JAP – Japan, LIT – Lithuania, SWE – Sweden, SLO – Slovenia). Taxonomy and nomenclature follows Viidalepp (1996) with the exception of using the tribal name Boarmiini instead of Cleorini (following Holloway (1993)).

Species	Voucher Code	Collection site and year	Collector	28S D1	28S D2	EF-1 a	Wgl	COI	GAPDH	RpS5	IDH
Drepanidae											
<i>Drepana lacertinaria</i> (Linnaeus)											
<i>Drepana curvatula</i> (Borkhausen, 1790)	NS48	SWE ¹ FIN, Hanko, 2002	K. Ruohomäki	AF178882 ¹	AF178906 ¹	-	AY948512	AY948539	GU580766	GU580820	GU580656 GU580849
Geometridae											
Archiclerinae											
<i>Archicleris parthenias</i> (Linnaeus, 1761)	NW107-1	SWE, Stockholm, 2003	N. Wahlberg	AF178897 ¹	AF178897 ¹	DQ018899 ²	DQ018869 ²	DQ018928 ²	EU141485 ²	EU141381 ²	EU141539 ²
Alsophilinae											
<i>Alsophila aescularia</i> (D. & S. 1775)	NS07	EST, Saaremaa, 2002	E. Õunap	GU580695	GU580726	GU580794	GU593332	GU580755	-	-	GU580856
Larentiinae											
<i>Opeolithera brumata</i> (Linnaeus, 1758)	NS29	FIN, Turku, 2000	K. Lempa	AF178891 ¹	AF178915 ¹	AY948488 ³	AY948516 ³	GU580781	-	-	-
<i>Epirrita autumnata</i> (Borkhausen, 1794)	NS22	FIN, Masku, 2000	K. Ruohomäki	AY948443 ³	AY948463 ³	AY948485 ³	AY948513 ³	EF206689 ⁴	GU580828	-	GU580864 GU580860
<i>Perizoma didymatum</i> (Linnaeus, 1758)	NS14	FIN, Seili, 2001	K. Ruohomäki	AY948448 ³	AY948469 ³	AY948492 ³	AY948520 ³	GU580784	-	-	-
Sterrhinae											
<i>Idaea stramineata</i> (Borkhausen, 1794)	NS02	EST, Kauksi, 1998	T. Tammaru	AF178889 ¹	AF178913 ¹	AY948507 ³	AY948534 ³	GU580773	-	GU580657	GU580851
<i>Scopula immorata</i> (Linnaeus, 1758)	NS01	EST, Karilatsi, 2002	T. Tammaru	AF178886 ¹	AF178910 ¹	AY948508 ³	AY948535 ³	GU580787	-	-	GU580850
Geometrinae											
<i>Jodis putata</i> (Linnaeus, 1758)	NS04	EST, Rannu, 2004	T. Tammaru	GU580692	GU580723	GU580812	GU593342	GU580776	-	-	GU580853
<i>Geometra papilionaria</i> (Linnaeus, 1758)	NS03	EST, Avinurme, 2004	T. Tammaru	GU580691	GU580722	GU580809	GU593343	GU580772	GU580821	-	GU580852
Ennominae											
Abraxini											
<i>Abraxas sylvata</i> (Scopoli, 1763)	NS24	FIN, Hanko, 2004	K. Ruohomäki	GU580704	GU580734	GU580800	GU593336	GU580762	GU580830	GU580668	-
<i>Lomasipilis marginata</i> (Linnaeus, 1758)	NS42	EST, Avinurme, 2005	T. Tammaru	GU580718	GU580748	GU580813	-	GU580777	GU580844	GU580685	GU580877
Caberini											
<i>Lomographa bimaculata</i> (Fabricius, 1829)	NS09	FIN, Nauvo, 2003	K. Ruohomäki	GU580697	GU580728	GU580814	-	GU580778	-	-	GU580857
<i>Cabera pusaria</i> (Linnaeus, 1758)	NS41	EST, Avinurme, 2004	T. Tammaru	GU580717	GU580747	GU580799	-	GU580761	-	GU580684	GU580876
Theriini											
<i>Theria rupicaprarria</i> (D. & S. 1775)	NS10	SWE, Karlskrona, 2002	B. Henriksson	GU580698	GU580729	GU580819	-	GU580789	-	GU580661	GU580858
Ennomini											
<i>Ennomos fuscantaria</i> (Haworth, 1809)	NS19	FIN, Turku, 2000	K. Ruohomäki	EF206659 ⁴	EF206667 ⁴	EF206674 ⁴	GU593335	EF206682 ⁴	GU580826	GU580665	GU580861
Colotoini											
<i>Colotois pennaria</i> (Linnaeus, 1761)	NS05	FIN, Hanko, 2003	K. Ruohomäki	GU580693	GU580724	GU580802	-	GU580764	-	GU580658	GU580854
Campaeini											
<i>Hylaea fasciaria</i> (Linnaeus, 1758)	NS45	EST, Karilatsi, 2005	T. Tammaru	GU580720	GU580750	GU580805	-	GU580768	GU580846	GU580688	GU580880
Macarini											
<i>Pygmaea fusca</i> (Thunberg, 1782)	NS13	FIN, Utsjoki, 2002	K. Ruohomäki	GU580699	GU580730	GU580817	GU593334	GU580786	GU580822	-	GU580859
<i>Semiothisa clathrata</i> (Linnaeus, 1758)	NS40	EST, Karilatsi, 2005	T. Tammaru	GU580716	GU580746	GU580818	-	GU580788	GU580843	GU580683	GU580875
<i>Itame loritaria</i> (Eversmann, 1837)	NS16	EST, Saare, 2004	I. Taal	GU580700	-	GU580811	-	GU580775	-	-	-
<i>Itame brumata</i> (Thunberg, 1784)	NS17	EST, Karilatsi, 2004	T. Tammaru	GU580701	GU580731	GU580810	-	GU580774	GU580824	GU580663	-
Gnophini											
<i>Siona lineata</i> (Scopoli, 1763)	NS44	EST, Karilatsi, 2005	T. Tammaru	EF206663 ⁴	EF206671 ⁴	EF206678 ⁴	GU593340	EF206686 ⁴	-	GU580687	GU580879
<i>Kentrographos ambigua</i> (Duponchel, 1830)	NS47	EST, Täheva, 2004	T. Tammaru	EF206664 ⁴	EF206672 ⁴	EF206679 ⁴	-	EF206687 ⁴	GU580848	GU580690	GU580882
<i>Cleodes lichenaria</i> (Hufnagel, 1767)	NS35	EST, Tõstamaa, 2005	T. Tammaru	EF206662 ⁴	EF206670 ⁴	EF206677 ⁴	-	EF206685 ⁴	GU580838	GU580678	GU580873
<i>Ematurga atomaria</i> (Linnaeus, 1758)	NS43	EST, Karilatsi, 2005	T. Tammaru	GU580719	GU580749	GU580806	-	GU580769	GU580845	GU580686	GU580878
Angeronini											
<i>Angeronia prunaria</i> (Linnaeus, 1758)	NS46	EST, Karilatsi, 2005	T. Tammaru	GU580721	-	GU580795	GU593341	GU580756	GU580847	GU580689	GU580881
Bupalini											
<i>Bupalus piniaria</i> (Linnaeus, 1758)	NS27	EST, Avinurme, 2004	T. Tammaru	GU580706	GU580736	GU580798	GU593339	GU580760	GU580833	GU580671	-
Boarmiini											
<i>Peribatodes secundaria</i> (D. & S. 1775)	NS38	EST, Ruhnu, 2005	T. Tammaru	EF206661 ⁴	EF206669 ⁴	EF206676 ⁴	-	EF206684 ⁴	GU580841	GU580681	-
<i>Arichanna tetrica</i> (Butler, 1878)		JAP ⁵		AB265528 ⁵	-	AB265455 ⁵	-	-	-	-	-
<i>Arichanna melanaria</i> (Linnaeus, 1758)	NS37	EST, Karilatsi, 2005	T. Tammaru	GU580714	GU580744	-	-	GU580758	GU580840	GU580680	GU580874

(continued on next page)

Table 1 (continued)

Species	Voucher Code	Collection site and year	Collector	28S D1	28S D2	EF-1a	Wgl	COI	GAPDH	RpS5	IDH
<i>Alcis repandata</i> (Linnaeus, 1758)	NS39	EST, Avinurme, 2004	T. Tammaru	GU580715	GU580745	GU580793	-	GU580754	GU580842	GU580682	-
<i>Heterarmia dissimilis</i> (Staudinger, 1897)		JAP ⁵		AB265539 ⁵	-	AB265466 ⁵	-	-	-	-	-
<i>Xerodes rufescens</i> (Motschulsky, 1861)		JAP ⁵		AB265556 ⁵	-	AB265483 ⁵	-	-	-	-	-
<i>Milionia zonea</i> (Moore, 1888)		JAP ⁵		AB265543 ⁵	-	AB265470 ⁵	-	-	-	-	-
<i>Hypomecis punctinervis</i> (Scopoli, 1763)	NS18	EST, Avinurme, 2004	T. Tammaru	EF206660 ⁴	EF206668 ⁴	EF206675 ⁴	-	EF206683 ⁴	GU580825	GU580664	-
<i>Deileptenia ribeata</i> (Clerck, 1759)	NS23	EST, Avinurme, 2004	T. Tammaru	GU580703	GU580733	GU580803	-	GU580765	GU580829	GU580667	GU580865
<i>Cleora cinctaria</i> (D. & S. 1775)	NS25	FIN, Raisio, 2003	K. Ruohomäki	GU580705	GU580735	GU580801	GU593337	GU580763	GU580831	GU580669	GU580866
<i>Paradarisa consonaria</i> (Hübner, 1799)	NS34	EST, Nigula, 2005	E. Öunap	GU580712	GU580742	GU580816	-	GU580783	GU580837	GU580677	-
<i>Aethalura punctulata</i> (D. & S. 1775)	NS36	EST, Nigula, 2005	E. Öunap	GU580713	GU580743	GU580790	-	GU580751	GU580839	GU580679	-
<i>Ectropis crepuscularia</i> (D. & S. 1775)	NS21	FIN, Hanko, 2002	K. Ruohomäki	GU580702	GU580732	GU580804	-	GU580767	GU580827	GU580666	GU580863
Bistonini											
<i>Biston stratararius</i> (Hufnagel, 1767)	NS33	FIN, Parainen, 2002	H. Takanen	GU580711	GU580741	GU580797	-	GU580759	GU580836	GU580676	GU580872
<i>Biston betularius</i> (Linnaeus, 1758)	NS26	EST, Karilatsi, 2004	T. Tammaru	EF206658 ⁴	EF206666 ⁴	EF206673 ⁴	GU593338	EF206681 ⁴	GU580832	GU580670	GU580867
<i>Lycia hirtaria</i> (Clerck, 1759)	NS15	EST, Avinurme, 1999	T. Tammaru	AY948461 ³	AY948482 ³	AY948509 ³	AY948536 ³	GU580779	GU580823	GU580662	-
<i>Lycia lapponaria</i> (Boisduval, 1840)	NS08	FIN, Masku, 2000	K. Ruohomäki	GU580696	GU580727	GU580815	GU593333	GU580780	-	GU580660	-
<i>Apocheima hispidarium</i> (D. & S. 1775)	NS28	LIT, Lazdijai, 2002	G. Svitra	GU580707	GU580737	GU580796	-	GU580757	-	GU580672	GU580868
<i>Agrotis aurantaria</i> (Hübner, 1799)	NS06	FIN, Nauvo, 2001	K. Ruohomäki	GU580694	GU580725	GU580791	-	GU580752	-	GU580659	GU580855
<i>Agrotis margaritaria</i> (Fabricius, 1776)	NS32	EST, Saaremaa, 2002	E. Öunap	GU580710	GU580740	GU580792	-	GU580753	GU580835	GU580675	GU580871
<i>Phigaliohybernia fulviflula</i> Inoue, 1942		JAP ⁵		AB265549 ⁵	-	AB265476 ⁵	-	-	-	-	-
<i>Phigalia pilosaria</i> (D. & S. 1775)	NS11	EST, Avinurme, 2005	T. Tammaru	AY948462 ³	AY948483 ³	AY948510 ³	AY948537 ³	GU580785	-	-	-
<i>Palaearctia merricata</i> (Dyar, 1903)	NS12	USA, Maryland, 2000	T. Tammaru	AY948463 ³	AY948484 ³	AY948511 ³	AY948538 ³	GU580782	-	-	-
<i>Erannis defoliaria</i> (Clerck, 1759)	NS20	FIN, Hanko, 2003	K. Ruohomäki	EF206665 ⁴	-	EF206680 ⁴	-	EF206688 ⁴	-	-	GU580862
<i>Erannis golda</i> (Djakonov, 1929)		JAP ⁵		AB265537 ⁵	-	AB265464 ⁵	-	-	-	-	-
<i>Erannis tilitaria</i> (Harris, 1841)	NS30	USA, Michigan, 2005	G. Balogh	GU580708	GU580738	GU580808	-	GU580771	GU580834	GU580673	GU580869
<i>Erannis ankeraria</i> (Staudinger, 1861)	NS31	SLO, Podgorje, 2004	S. Gomboc	GU580709	GU580739	GU580807	-	GU580770	-	GU580674	GU580870

-, not available

¹ Abraham et al., 2001² Wahlberg & Wheat, 2008³ Snäll et al., 2007⁴ Viidalepp et al., 2007⁵ Yamamoto & Sota, 2007

2.2. DNA extraction, PCR and sequencing

Prior to DNA extraction, samples were preserved in ethanol or stored dried or frozen. Genomic DNA was extracted using the DNeasy™ Tissue Kit (Qiagen) according to the manufacturer's instructions. Either the entire individual or a part of it was used for extraction. We amplified EF-1 α , wgl and segments D1 and D2 of 28S rRNA using the same protocols and primers described in Snäll et al. (2007). Amplification of the COI fragment was carried out with LCO2198 and HCO1490 primers (Folmer et al., 1994). For the remaining gene regions (IDH, GAPDH and RpS5), we followed the protocols of Wahlberg and Wheat (2008). PCR reactions were performed with 5–100 ng of DNA template, 0.1 μ M of each primer, 200 μ M of each dNTP, 1 U of DyNAzyme™ II DNA polymerase (Finnzymes Oy) in 1 \times DyNAzyme™ buffer supplemented with 1.5 mM MgCl₂ in a PTC-100 Thermal Cycler (MJ Research Inc.) or in an Eppendorf Mastercycler® gradient (Perkin-Elmer Corp.). PCR conditions included an initial denaturation at 95 °C for 5 min followed by 35 cycles of 30 s at 95 °C, 30 s–1.5 min at 49–57.5 °C depending on the gene fragment and the primer pair, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.). The sequencing was performed with an automatic ABI PRISM™ 377 or 3130xl DNA Sequencer using the ABI PRISM® Big-Dye™ Terminator chemistry (Applied Biosystems, Foster City, CA). For some taxa, PCR amplification products and sequence data were not obtained for all gene fragments (see Table 1).

2.3. Phylogenetic analysis

The sequences were initially edited with Sequencher™ v.3.1.1 (Gene Codes Corp.) or BioEdit (Hall, 1999). The protein coding genes were trivial to align, with no indel events inferred except in the case of wgl, where some codon insertions and deletions were inferred (see Section 3). Alignment of the 28S fragments proved difficult, as found in Snäll et al. (2007). Initial analyses with POY (Wheeler et al., 2003) using various combinations of parameter values (see Snäll et al., 2007 for details) suggested that there were clearly defined conservative regions, which produced identical implied alignments regardless of parameter values used, as well as hypervariable regions, which produced highly divergent implied alignments depending on the parameter values used. For further analyses, we deleted the hypervariable regions and used the resulting conservative alignment in combination with the other gene regions.

The seven gene regions (28S D1 and D2 were combined) and the morphological data were analysed separately and combined using parsimony. We searched for the most parsimonious cladograms for the equally weighted and unordered data matrices using a heuristic search algorithm in the program TNT (Goloboff et al., 2008). The data were subjected to 100 random addition rounds of successive Sectorial, Ratchet, Drift and Tree Fusing searches (Goloboff, 1999; Moilanen, 1999; Nixon, 1999). We estimated the support for the resulting clades using bootstrap analyses (1000 pseudoreplicates in TNT using 10 random additions and TBR).

In the analysis of morphological data, 89 morphological characters of adult moths and pupae for ennomines were coded (Supplementary material 1), and analysed using the program NONA (Goloboff, 1998) with equal weights. The data were also subjected to a bootstrap analysis, with 1000 pseudoreplicates, each with 10 random additions and two trees kept. Trees were visualised with the program Winclada (Nixon, 2002).

We also analysed the molecular data separately as well as the combined morphological and molecular data matrix using Bayesian phylogenetic methods in the program MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The molecular data partitions were ana-

lysed with mixed models, i.e. parameters for the GTR + Γ model were estimated separately for the seven gene regions using the “unlink” command in MrBayes. The morphological data were subjected to a rate variable model (Lewis, 2001). The analyses were run for 10 million generations with the chain being sampled every 1000 generations. The point of convergence was determined visually by plotting the log likelihood of the data against the number of generations and then the “burn-in” period was discarded. The posterior probabilities for clades were summarized through a majority rule consensus tree of the sampled trees. The molecular data on its own was subjected to the same analysis in a combined analysis framework.

We estimated times of divergence using the program BEAST v1.4.6 (Drummond et al., 2006), which implements a relaxed molecular clock in a Bayesian framework and estimates a topology simultaneously. For this analysis we combined the molecular data and analysed it as one partition with the GTR + Γ model and a relaxed clock allowing branch lengths to vary according to an uncorrelated Lognormal distribution (Drummond et al., 2006). To avoid long branch artefacts, we did not include the Drepanidae sequences in the analysis. The tree prior was set to the Yule process, all other priors were left to the defaults in BEAST. In order to estimate times of divergences, we calibrated the age of Geometridae according to the results reported in Yamamoto and Sota (2007), i.e. 54.4 million years with a standard deviation of 4.0 million years. A wide SD was chosen in order to account for the error inherent in using secondary calibration points (Graur and Martin, 2004). Parameters were estimated using two independent runs of 10 million generations each (with a pre-run burn-in of 100,000 generations), and sampled every 1000 generations. Convergence was checked in the Tracer v1.4.6 program and summary trees were generated using TreeAnnotator v1.4.6, both part of the BEAST package.

3. Results

3.1. Sequence data

Sequencing of the seven nuclear and one mitochondrial gene fragments produced a total of 4886 bp of aligned sequence data from Ennominae and outgroup species for phylogenetic analysis. The basic statistics of each gene region are given in Table 2. Insertion/deletion events were inferred for the 28S fragments, as well as wgl, where two codon insertions were found in the three species of Operophterini (see Snäll et al., 2007) and two codon deletion events were inferred for *Geometra* and *Jodis*. Wgl failed to amplify for almost all Ennominae species (Table 1), suggesting that it is not a suitable marker for this subfamily. The new gene regions (IDH, GAPDH and RpS5) amplified relatively well from the geometrid samples, mainly failing in cases where the DNA had degraded. The new gene regions appear thus to be suitable for molecular systematic studies of Geometridae.

3.2. Phylogenetic relationships

Our reconstruction of the phylogenetic relationships of Ennominae revealed a tree with several unambiguous patterns (Fig. 1). The sampled species of the geometrid subfamilies Larentiinae, Sterrhinae and Geometrinae formed well-supported monophyletic groups with bootstrap values of 94–100 and posterior probabilities of 1. The subfamily Archiearinae, represented just by one species in the present study, stands as its own lineage. The subfamily Ennominae also formed a monophyletic group, however, it included the representative of the traditional subfamily Alsophilinae, *Alsophila aescularia*. Our phylogenetic hypothesis suggests that the sister group of Ennominae is Geometrinae, with Archiearinae being sister

Table 2
Basic statistics for genes used in this study.

Gene	No. of sites	No. aligned sites	No. variable	No. informative	No. of taxa sequenced
28S D1	329–333	333	47	25	49
28S D2	444–479	432*	177	144	49
COI	676	676	254	224	49
EF-1 α	1021	1021	349	293	49
GAPDH	691	691	259	222	30
RpS5	617	617	222	185	36
IDH	710	710	320	280	35
wgl	394–406	406	182	155	22

* Hypervariable regions removed.

to these two. At the subfamily level, our results thus coincide with those of Yamamoto and Sota (2007), including subsuming *Alsophilinae* in *Ennominae*.

Within *Ennominae*, two main clades emerged in both analyses, being well supported in the Bayesian analysis, but not in the parsimony analysis. These clades agree with the morphology-based division of *Ennominae* into the “ennomine” and “boarmiine” groups of genera (Forbes, 1948; Holloway, 1993). The clade corresponding to the “ennomine” group, which contained two species with flightless females, was further subdivided into two strongly supported subclades. Both species with flightless females, *Alsophila aescularia* and *Theria rupicaprararia*, fell into the same subclade though they clearly did not represent sister groups.

The clade corresponding to the “boarmiine” group as a whole was well supported in both analyses and comprised the representatives of the traditionally recognised tribes Boarmiini, Bistonini, Bupalini, Macariini and Abraxini. Macariini and Abraxini were sister groups to each other with strong support, and together sister to the rest of the “boarmiine” group in the Bayesian analysis. The clade formed by representatives of Bistonini, Boarmiini and Bupalini was characterized by polytomies in the strict consensus tree and the Bayesian majority rule consensus tree of the combined dataset (Fig. 1), as well as in each of the single gene analyses (see Supplementary material 2), indicating a lack of phylogenetic signal at these nodes. This implies that the question about monophyly of Bistonini could not be definitively resolved, i.e. the “traditional Bistonini” genera *Agriopsis*, *Apocheima*, *Biston*, *Erannis*, *Lycia*, *Paleacrita* and *Phigalia* may form a clade separate from the traditional Boarmiini as suggested by the morphological data (Fig. 1c), but the evidence for this is not strong (Fig. 1a, b and d) or even conflicting (Fig. 2) when molecular data is added. However, the molecular data convincingly show that if Bistonini is monophyletic, *Bupalus* and *Arichanna* should be included in the tribe.

3.3. Independent origins of female flightlessness and their timing

Despite of the poor resolution of the “boarmiine” group, it was possible to identify seven phylogenetically independent cases of the evolution of female flightlessness in the subfamily *Ennominae* (Fig. 1). In particular, within the ennomine clade, *Alsophila* and *Theria* clearly represent independent transitions to flightlessness, as does *Itame loricaria* in the boarmiine branch. Within the broad sense Boarmiini, there are four independent transitions as indicated by the pattern that sister groups of (1) *Agriopsis*, (2) *Erannis* and (3) *Lycia lapponaria* all have sister groups containing fully winged moths. Making the reasonable assumptions that the common ancestor of Boarmiini was a moth with fully winged females, and reversions to the winged state are improbable (see Section 4 below), the above situation also implies that the clade formed by the genera *Phigalia*, *Apocheima* and *Paleacrita* represents the fourth independent case of the evolution of flightlessness within Boarmiini s. lat.

The topology recovered in the BEAST analysis is much more resolved than in the previous analyses, although the resolved nodes have low posterior probabilities (Fig. 2). With the caveat that our estimates of absolute times of divergence are entirely dependent on the study by Yamamoto and Sota (2007), from which we took our calibration time, we find that the subfamily *Ennominae* began diversifying around the Eocene/Oligocene boundary, with several major divergences happening in the Oligocene. There appears to have been a period of rapid radiation in the “boarmiine” group around the Oligocene/Miocene boundary. Regardless of absolute times of divergence, it appears that the independent evolutionary events leading to wingless females have happened at very different times relative to each other. Apparently there was not just one single historical event that can be used to explain the evolution of this trait.

4. Discussion

4.1. Phylogenetic relationships

At the subfamily level, the present molecular phylogeny concurs very well with the relationships suggested by traditional geometrid systematics. In particular, our molecular data – confirming the results of Young (2006) and Yamamoto and Sota (2007) – strongly support the monophyly of each of the major geometrid subfamilies, i.e. Larentiinae, Sterrhinae, Geometrinae and *Ennominae*. Notably, this is a quite different situation compared to some other lepidopteran families, Noctuidae and Pyralidae in particular, (Lange et al., 2004; Mitchell et al., 2006) in which the incongruence between the molecular-based phylogeny and traditional systematics is particularly evident.

A finding of relevance at the level of subfamily taxonomy of Geometridae was that the representative of the traditionally recognized subfamily *Alsophilinae*, *Alsophila aescularia*, unambiguously grouped with *Ennominae* falling into the “ennomine” group and related to Colotoini + Campaeini (exact relationship to these two is ambiguous). The classification of *Alsophila* has been problematic. Herbulot (1962) raised this taxon to subfamily level, which has, however, been questioned (Holloway, 1996). Indeed, *Alsophila* possesses distinctive taxon specific characters but also shares both “ennomine” and “boarmiine” like morphological features (Young, 2006). The placement of *Alsophila* in the ennomine branch of *Ennominae* is consistent with the results of Yamamoto and Sota (2007) but disagrees with the findings of both Abraham et al. (2001) and Young (2006), which indicate the close relationship of *A. aescularia* with the boarmiine genus *Agriopsis*. However, we believe that a mistake had happened in the Abraham et al. (2001) analysis, as their 28S D2 sequence for *Alsophila* is identical to their *Agriopsis aurantiaria*. We sequenced both species independently for the same gene, and found the 28S D2 sequence for *Alsophila* to be different, while the *A. aurantiaria* sequence was identical to that in Abraham et al. (2001).

The current molecular analysis provides full support to the morphology-based division of *Ennominae* into “ennomine” and

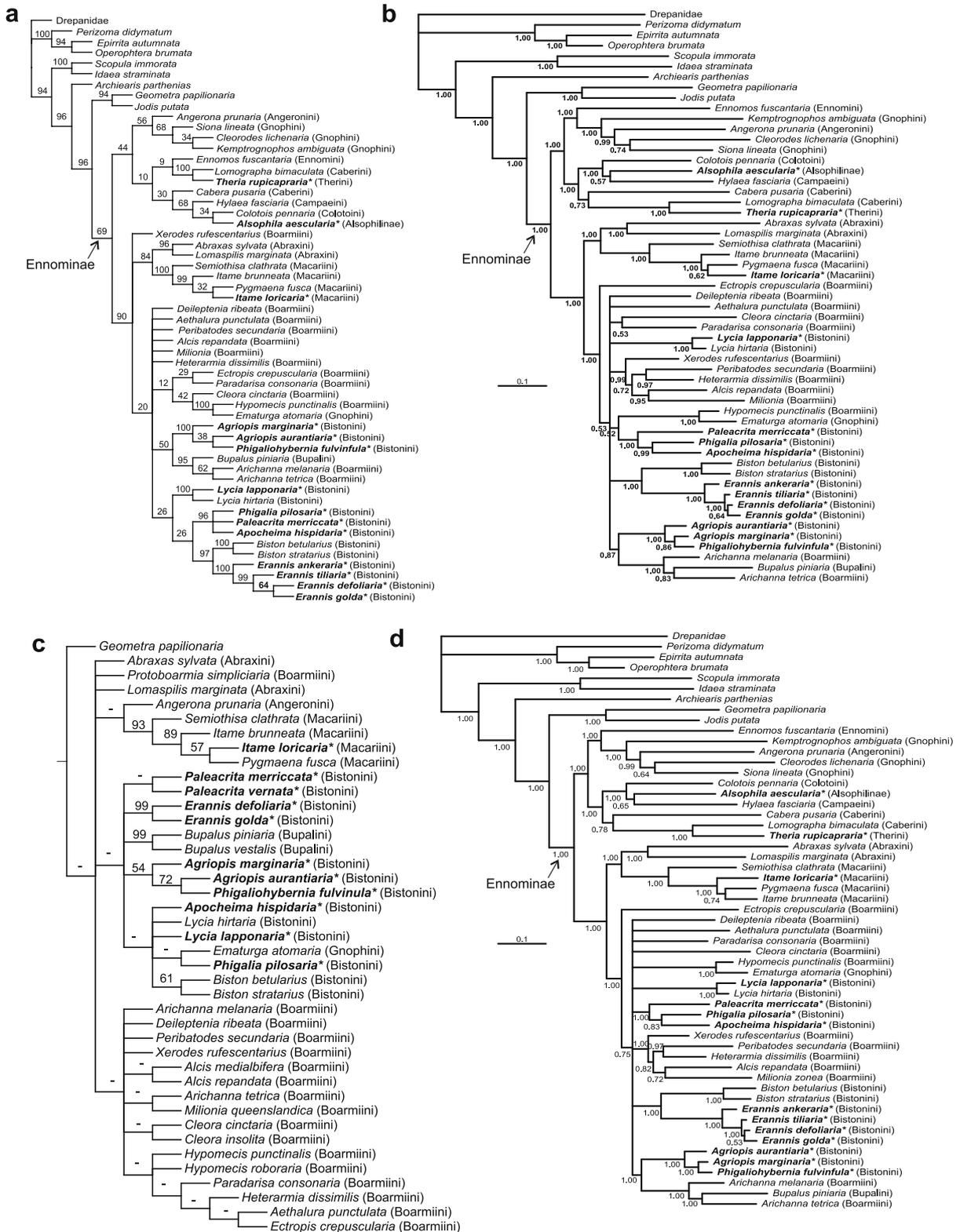


Fig. 1. (a) Strict consensus of six trees found in the parsimony analysis of the combined morphological and molecular dataset under equal weights (length = 10,485, CI = 0.297, RI = 0.391). Numbers above branches are bootstrap proportions. (b) Majority rule consensus of 18,002 trees sampled in the Bayesian analysis of the combined dataset. Numbers to the left of each node are the posterior probabilities of those nodes. (c) Strict consensus of 55 trees found for the morphological dataset under equal weights (length = 622, CI = 0.20, RI = 0.51). Numbers above branches are bootstrap proportions. (d) Majority rule consensus of 18,002 trees sampled in the Bayesian analysis of the molecules only dataset. Numbers to the left of each node are the posterior probabilities of those nodes. Species with wingless females are indicated by bold lettering and asterisks. Tribal classification of Ennominae in accordance with Table 1.

“boarmiine” groups (Forbes, 1948; Holloway, 1993; Viidalepp et al., 2007). The last clade includes Abraxini, Macariini and Boarmiini in

the broad sense. Also, the sometimes questioned (Holloway, 1993) relatedness of *Lomaspilis* to typical Abraxini is clearly confirmed.

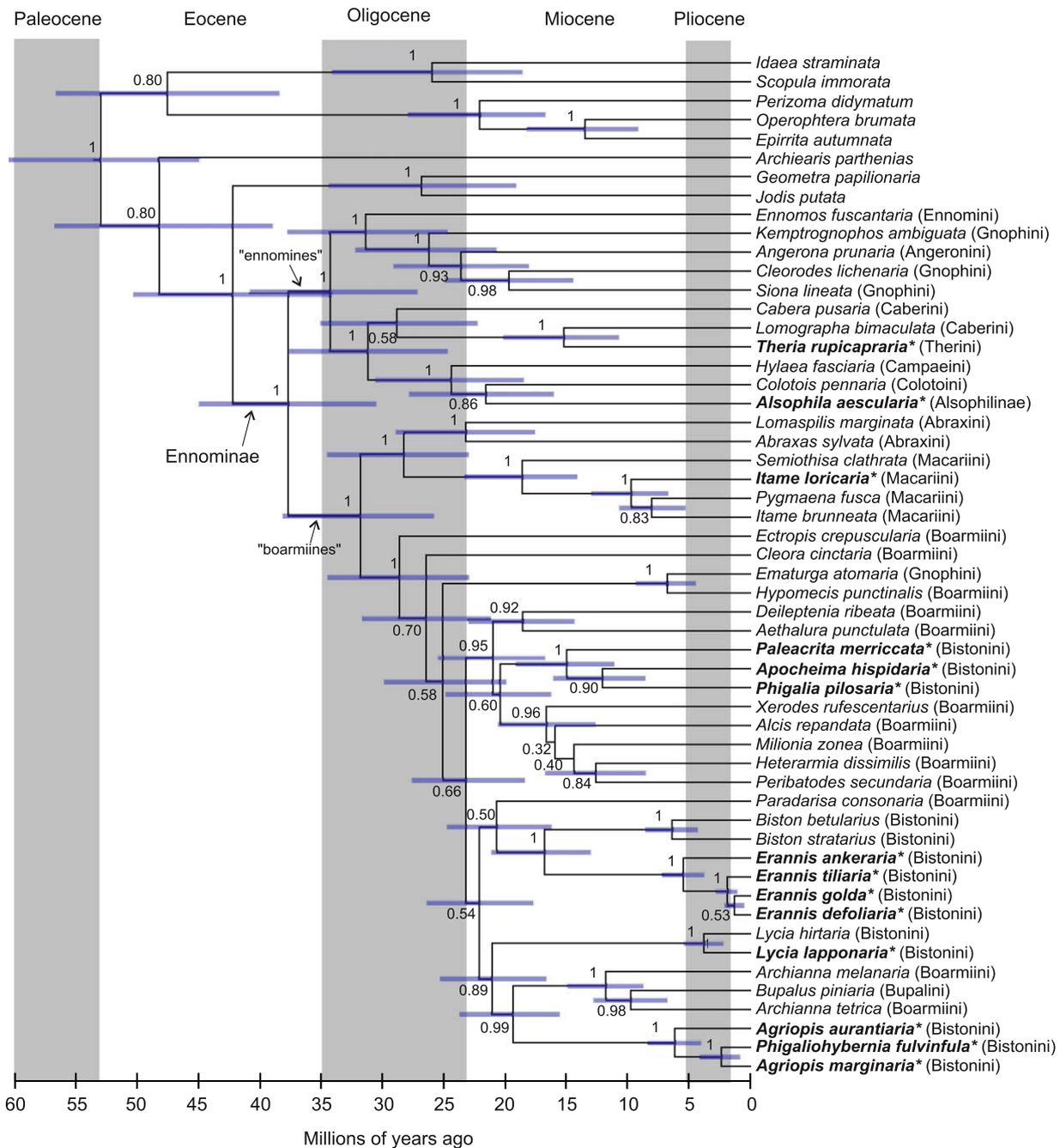


Fig. 2. Estimated times of divergence based on a relaxed clock analysis in BEAST and the molecular data. Bars on nodes represent the 95% credibility intervals for divergence time estimates of each node, numbers to the left of each node are the posterior probabilities for those nodes. Species with wingless females are indicated by bold lettering and asterisks.

The largely unresolved part of the phylogeny coincides with the tribe Boarmiini in its broadest sense (Holloway, 1993). Consequently, indeed, the crucial question about the relationship between the tribes Boarmiini and Bistonini remained unresolved in the framework of the present work. In the framework of the traditional morphology-based taxonomy, Bistonini and Boarmiini have been distinguished by a number of putative synapomorphies (see Viidalepp et al., 2007, for more details). In our formal analysis of the morphological data, however, we were unable to provide definitive support for the dichotomy though there was some indication of it. Importantly, we were unable to find any unique synapomorphies for either Bistonini or Boarmiini s. str. in the morphological data set (Appendix 2). Moreover, it has to be considered that a

number of traits characteristic of Bistonini may be independently evolved adaptations related to the winter moth syndrome which reduces the value of morphological data in this particular context. The putative adaptations range from the well understood reduction of proboscis (winter moths do not feed as adults) to less straightforward ones, e.g. the plumose or tufted antennae which, are, however, also characteristic of winter-flying geometrids of arid environments (Stüning and Viidalepp, in preparation), and a number of unrelated winter-flying moths (*Pterophora*, *Poecilocampa* etc.). Similarly, also at a more general level, there appeared to be little phylogenetic signal in the morphological data. The quite “chaotic” nature of the message delivered by the morphological data was more formally supported by the results of the joint

analysis of morphological and DNA sequence data not differing from the purely DNA based results (Fig. 1). This indicates that the occasionally conflicting morphological evidence was overridden by the phylogenetic information from the analysed sequences.

In the case of Bistonini and Boarmiini, it appears to be reasonable to recognise one large tribe, like Holloway's (1993) Boarmiini in the broad sense, as our rather extensive data was not able to resolve either of these tribes as monophyletic groups with respect to each other. Further studies are needed to definitively solve the question though the possibility of a true polytomy cannot be ruled out. Our estimates of times of divergence (Fig. 2) do suggest that the major lineages in Boarmiini in the broad sense diverged in quick succession from each other during the Oligocene. Thus, despite data from seven gene regions and a total of 4886 bp, we find that the lineages remain unresolved due to lack of phylogenetic information at those nodes.

Our results also show that the two taxonomically problematic genera, *Ematurga* and *Bupalus* should be subsumed within the broad concept of Boarmiini (Fig. 1). *Ematurga* shows a so far unanticipated close relationship with *Hypomecis* based on the molecular data (Fig. 1). The sister group relationship between these genera was a robust result as this pattern appeared in all single gene phylogenetic trees (except IDH, for which *Hypomecis* did not amplify, Supplementary material 2). Pierce (1914) placed the genus *Ematurga* in the beginning of his Boarmiinae list, while Wehrli (1953) lists it under unplaced genera at the “tail” of ennomines. Similarly, the taxonomic history of *Bupalus* has not been straightforward. The tribe Bupalini was erected by Herbulot, 1963, while Pierce (1914) associated it with Cymatophoridae (now placed in Drepanidae), and Wehrli (1953) retained it unplaced within Ennominae. It may well be the case that the diurnal activity of adults of both *Ematurga* and *Bupalus* has been the main reason for their morphological differentiation from other members of Boarmiini which has also caused the tradition to classify them as distinct from other related genera.

4.2. Findings of lower level taxonomic relevance

In addition to the broader scale patterns discussed above, some findings of lower level taxonomic importance should be discussed (inference based on Fig. 1) even if actual taxonomic changes is beyond the scope of the current manuscript. First, *Cleorodes lichenaria* appears to belong in the tribe Gnophini (in the broad sense) and not to Boarmiini as traditionally assumed (discussed in detail in Viidalepp et al., 2007). Second, according to our results, *Erannis* and *Agriopis* are not closely related to each other, and the highly similar appearance of these moths (formerly considered congeneric also based on similar structure of male antennae) should thus be viewed as a result of convergent evolution (most likely, wing morphology enabling active flight at low temperatures, and mimicking yellow autumn leaves). Furthermore, *Erannis ankeraria* is confirmed to belong to *Erannis* (Scoble, 1999) and not to *Agriopis* as sometimes proposed (Wehrli, 1940). Third, it does not appear to be justified to distinguish *Phigaliohybernia* as a separate genus and thus the East Palaearctic *P. latifasciaria* Beljaev, 1996 and *P. fulvifula* should be treated as members of *Agriopis*. Moreover, the sometimes disputed (Wehrli, 1940) close relationship between *Phigalia* and *Apocheima* should be considered certain. Finally, our results concur with the suggestion of Kullberg et al. (2001) to transfer *Pygmaena fusca* to *Itame*.

4.3. Evolution of flightlessness in Ennominae females

As based on the results of our phylogenetic analysis, at least seven independent transitions to female flightlessness have occurred in the geometrid subfamily Ennominae, four of them in the tribe Boarmiini in the broad sense. This differs considerably from the

suggestion of Hunter (1995) of only two origins of wing reduction in this group. In six out of seven cases, female winglessness is associated with winter flight (*I. loricaria* being the single exception), and larval polyphagy (not in *T. rupicaprarria*). This suggests that the evolution of the flightless phenotype is a frequent and non-random correlate of certain ecological factors which, along with the relatively high number of evolutionary transitions to flightlessness in holarctic forest Geometridae (two more in Larentiinae, Snäll et al., 2007), opens the way to phylogenetic comparative analyses which are bound to provide important insights into evolutionary ecology of forest insects. Formal comparative analyses (including ancestral state reconstruction) are beyond the scope of the present paper, partly because the values of several ecologically important traits are not readily available in standardised format for a number of species. Nevertheless, some emerging trends will be discussed below at the qualitative level, primarily to provide hypotheses for further rigorous analyses.

The tribe Bistonini (or even Boarmiini in the broad sense) comprises the group of geometrids in which wing reduction has evolved more frequently than in any comparable clade in the family Geometridae. This is consistent with one of the main aspects of our “adaptive story” of the evolution of flightlessness in forest moths (Snäll et al., 2007): some phylogenetic groups are predisposed for flightlessness to evolve. These predisposing evolutionary events, i.e. events that have preceded the wing reduction, most probably include the colonisation of stable forest habitats and the evolution of a set of permissive traits: larval polyphagy, spring-feeding larvae, the egg or pupa as the overwintering stage, and late/early adult flight season. These traits reduce the importance of both oviposition site selection and adult feeding, allowing flightlessness to evolve (Snäll et al., 2007). The reconstructed phylogeny of Ennominae is consistent with the implication that the ancestor of the wing-reduced species within Boarmiini s. lat. has been a winged but slow flying (especially females) forest moth feeding polyphagously on deciduous trees. Indeed, most of the winged representatives of the boarmiine clade (typical “Boarmias”: *Ectropis*, *Alcis*, *Hypomecis*, *Cleora* etc.) match well with this description despite not forming a common clade. These traits could therefore be seen as inherited from a common ancestor of the tribe. Notably, the representatives of the “ennomine” clade in the temperate forest zone are broadly characterised by a much more limited tendency towards polyphagy, and towards reduction in female mobility which may thus explain less frequent evolutionary switches to female flightlessness.

The above discussion is, however, based on the premise that the transition from a wingless form back to a winged form has not been likely in insect evolution. Indeed, traditionally, the re-evolution of such complex traits like wings has been considered unfeasible (West-Eberhard, 2003) until quite recently the study of Whiting et al. (2003), showed that the ancestor of stick insects was wingless and that wings have re-evolved four times within this insect group. There has been opposition to this conclusion (e.g. Trueman et al., 2004), and no other phylogeny-based cases of re-evolving wings have yet appeared. Even if the general idea about the impossibility of complex trait reversals has recently been challenged (Collin and Miglietta, 2008), we are strongly inclined to think (Snäll et al., 2007) that winter moths represent a case in which re-evolving flight ability is particularly improbable. This because, in this group, the flightlessness affects not only the wings but the whole body composition. Flightless females typically possess a large abdomen and small thorax, and the reversal to the flying phenotype would require various substantial changes in body composition and behaviour (discussed in detail in Snäll et al., 2007). Further progress in the reconstruction of lepidopteran phylogeny would certainly provide us also more evidence bearing to this question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.025.

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