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Phylogenetic relationships of selected European Ennominae (Lepidoptera: Geometridae)

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Abstract. This study reports the results of a molecular phylogenetic analysis of thirty three species of Ennominae (Lepidoptera: Geometridae). The aim of this analysis was to determine the phylogenetic affinities of 13 European species not previously studied using these methods. Fragments of seven nuclear genes, elongation factor 1 alpha (*EF-1α*), wingless (*wgl*), isocitrate dehydrogenase (*IDH*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S5 (*RpS5*) and expansion segments *D1* and *D2* of the 28S rRNA gene and fragment of one mitochondrial gene, cytochrome oxidase subunit I (*COI*), were used. In the analysis using Bayesian phylogenetic inference, original gene sequences of the target species were combined with a larger data matrix of 20 species of Ennominae, used in a previous study (Wahlberg et al., 2010, *Mol. Phylogenet. Evol*. **55**: 929–938). Most notably, the results indicate that the representatives of the genera *Cepphis*, *Plagodis*, *Pseudopanthera* and *Selenia* form a well-supported monophyletic group which appeared as the sister clade to the rest of the "ennomine" group of tribes. On the other hand, *Crocallis* and *Opisthograptis* group together with *Ennomos*. These results conflict with previous tribal subdivisions of the subfamily pointing to the need to reconsider the concepts of Ennomini and Ourapterygini. Within the tribe Macariini, the genus *Macaria* appears to be more closely related to *Itame* (=*Speranza*) than to *Chiasmia clathrata*. The emerging phylogenetic tree of Ennominae suggests only a limited phylogenetic inertia in body size making this group a promising target for comparative studies on this central life history trait and its correlates.

INTRODUCTION

The largest subfamily of Geometridae, the Ennominae (about 45% of all Geometridae: Minet & Scoble, 1999), is a well defined and uncontroversial group. However, there is no consensus about the taxonomic affinities of the numerous currently recognized tribes within the subfamily (Heppner, 2003). Within Ennominae, the genera 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 & Turčani, 2005; Viidalepp et al., 2007; Wahlberg et al., 2010). Beyond this major subdivision, the relationships among the numerous traditionally recognised tribes of Ennominae have remained largely uncertain (see Holloway, 1993 for a recent morphology-based hypothesis) and await a reassessment using contemporary methods of phylogenetic analysis.

In fact, there still are only few molecular-based studies on the phylogeny of geometrids. The first molecular phylogeny of a sample of geometrid species was published as recently as 2001 (Abraham et al., 2001). To date, there appear to be just three large-scale studies of molecular relationships: the one by Young (2006), based on a sample of mainly Tasmanian species; an analysis of a limited set of Japanese taxa (Yamamoto & Sota, 2007) and the most recent one addressing the evolution of female flightlessness among holarctic Ennominae (Wahlberg et al., 2010). There are, however, an increasing number of more focused taxon-specific molecular studies on geometrids (e.g. Snäll et al., 2007; Viidalepp et al., 2007; Õunap et al., 2008, 2009).

The goal of the present study was to establish phylogenetic affinities of 13 European species of Ennominae not studied earlier using molecular methods. Various life-history traits of these species and a number of other geometrids included in previous phylogenetic studies (Snäll et al., 2007; Wahlberg et al., 2010) are currently being studied (Javoiš et al., in prep.). Knowing the position of these taxa in the phylogenetic tree of the subfamily is a precondition for subsequent phylogenetically explicit comparative analyses. In particular, the great variability in body size, characteristic of this subfamily, is most promising in the context of further studies on the evolutionary ecology of body size. Although body size is a trait of central importance in life history studies (e.g. Roff, 1992), the selective forces determining optimal size in insects are poorly understood (e.g. Blanckenhorn, 2000; Tammaru et al., 2002; Gotthard, 2004). Phylogenetic comparative analyses appear most promising in this context.

For the phylogenetic analysis, 7 nuclear gene fragments [partial sequences of elongation factor 1 alpha (*EF-1α*), wingless (*wgl*), isocitrate dehydrogenase (*IDH*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S5 (*RpS5*) and expansion segments D1 and D2 of 28S rRNA gene] were sequenced in addition to one mitochondrial gene fragment, a partial sequence of

cytochrome oxidase subunit I (*COI*). The selection of markers follows that of Wahlberg et al. (2010), which allows the original sequence data obtained in this study to be combined with the larger data set used in Wahlberg et al's article. Based on the combined data matrix, a phylogenetic tree was derived using Bayesian phylogenetic inference. In addition to providing necessary information for forthcoming comparative analyses the results help to resolve several taxonomic ambiguities in the Ennominae.

MATERIAL AND METHODS

Species studied

In total, 33 Ennomine species were included in the present analysis (Table 1), 20 of which were used earlier to construct a preliminary phylogenetic tree for the Ennominae (Wahlberg et al., 2010) and provide the necessary reference framework. The remaining 13 taxa, however, had not previously been subjected to a rigorous phylogenetic analysis (Table 1). These 13 newly studied species represent both the "ennomine" (tribes Ennomini and Ourapterygini, sensu Viidalepp, 1996) and "boarmiine" branch (Macariini, Abraxini) of the subfamily. The specimens used in this study were collected from Estonia (Table 1), voucher specimens are housed in the Museum of Zoology, University of Tartu.

DNA extraction, PCR and sequencing

DNA was extracted from parts of dried specimens using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions for isolation of nucleic acids from mammalian tissue. A fragment of one mitochondrial (*COI*) and five nuclear protein-coding genes (*EF-1α, wgl, GAPDH, IDH, RpS5*) and two fragments (expansion segments *D1* and *D2*) of one nuclear ribosome gene (*28S rRNA*) were amplified. Primers for amplifying *GAPDH*, *IDH* and *RpS5* had universal tails on their 5' ends (see Regier & Shi, 2005; Wahlberg & Wheat, 2008) that allowed us to sequence the respective gene fragments using common sequencing primers T7 Promoter and T3 (Table 2). All other gene fragments were sequenced utilizing the same primers that were used for PCR (Table 2).

PCR was performed in a total volume of 20 µl, with the reaction mixture containing 1X BD Advantage 2 PCR buffer, 1U BD Advantage 2 Polymerase mix (BD Biosciences, San Jose, USA), 0.2 mM dNTP (Fermentas, Vilnius, Lithuania), 4 pmol of primers and 20–80 ng of purified genomic DNA. PCR was carried out in a Biometra T1 Thermocycler (Biometra, Göttingen, Germany), its conditions were an initial denaturation at 94°C for 2 min followed by 35–40 cycles of 30 s at 94°C, 30 s at 50–63°C depending on the gene fragment and the primer pair (Table 2), and 1 min at 68°C, and a final extension at 68°C for 7 min. PCR products were visualised on a 1.6% agarose gel and 10 µl of the PCR solution was treated with fast alkaline phosphatase and exonuclease I (Fermentas). DNA cycle sequencing was performed in a total volume of 10 µl using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Cycling conditions were: initial denaturation for 1 min at 96°C followed by 25 cycles of 10 s at 95°C, 15 s at 47–58°C and 4 min at 60°C. Both DNA strands were sequenced using 1.6 pmol of primers, and sequences were resolved using 3730xl DNA Analyzer (Applied Biosystems). For some taxa, PCR amplification products and sequence data could not be obtained for all gene fragments (see Table 1).

Phylogenetic analysis

Consensus sequences were created in Consed (Gordon et al., 1998) using sequence data from both DNA strands. Sequences were double-checked by eye, edited in BioEdit (Hall, 1999) and aligned in ClustalW (Thompson et al., 1994) using the default settings. The protein coding genes were trivial to align, with no indel events inferred except in the cases of *wgl* and *RpS5*, as one 3 bp deletion was found in both two genes of *Plagodis pulveraria*. The lengths of the fragments of protein-coding genes used in the phylogenetic analysis were 667 bp for *COI*, 1008 bp for *EF-1α*, 389 bp for *wgl*, 691 bp for *GAPDH*, 699 bp for *IDH* and 617 bp for *RpS5*. Alignment of the 28S fragments proved difficult, as had been shown earlier by Snäll et al. (2007), Õunap et al. (2008) and Wahlberg et al. (2010). The length of successfully sequenced fragments of D1 varied from 281–283 bp and the length of aligned data matrix was 283 bp. Three positions with indels were excluded from data matrix resulting in a 280 bp indel-free matrix. The alignment of D2 was more complicated, as the length of successfully sequenced fragments varied from 402–440 bp and the length of aligned data matrix was 450 bp. Of those positions 83 contained indels and were removed, resulting in a 367 bp indel-free data matrix. The total length of the combined data matrix was 4718 bp. GenBank accession numbers of both originally contributed and downloaded sequences are provided in Table 1.

All previous molecular phylogenetic studies have resolved Ennominae as a monophyletic entity (Abraham et al., 2001; Young, 2006; Yamamoto & Sota, 2007; Wahlberg et al., 2010). Therefore, monophyly of this group was assumed and as appropriate analytical methods are now available, no outgroups were used in this study in order to avoid possible long-branch attraction effects.

For phylogenetic analysis, data were divided into three partitions. First, *COI* as the single mitochondrial protein-coding gene was treated as a separate partition. Second, as expansion segments D1 and D2 of *28S* are different regions of the same rRNA gene and therefore share a similar evolutionary history, data from these gene fragments were concatenated and treated as a single partition. Third, as sequencing nuclear protein-coding genes proved difficult and failed on a number of occasions (Table 1) the data from all respective gene fragments were treated as one partition and failed regions were defined as missing characters. Modeltest 3.06 (Posada & Crandall, 1998) was used in PAUP*4.0b10 (Swofford, 1998) to search for the model of DNA substitution that best fitted the data for each partition. Beast v1.5.4 (Drummond & Rambaut, 2007) was used for the Bayesian estimation of phylogeny, implementing GTR + I + Γ model selected by Modeltest for each of the tree partitions and using relaxed molecular clock allowing branch lengths to vary according to an uncorrelated lognormal distribution (Drummond et al., 2006). To obtain an ultrametric tree, the age of the Ennominae was calibrated according to Wahlberg et al. (2010), i.e. 37.5 million years with a standard deviation of 6.5 million years. The TMRCA of *Hypomecis* + *Ematurga* clade was given a uniform prior distribution from 4 to 10 million years according to the same article. The tree prior was set to the Birth-Death process and all other priors were left to defaults in Beast. First, Bayesian MCMC was run over 30 million generations, sampling every 1000th generation. Thereafter, suggestions by Beast for improving the analysis were taken into account and four further MCMC runs (one for 50 million and three for 30 million generations, sampling every 1000th generation) were performed. The results of these four analyses were combined and inspected with Tracer v1.5. The first 10% of the sampled trees were discarded as "burn-in" from each of the three analyses and the remaining

TABLE 1. Details of the specimens used in the molecular analysis. Collecting site (EST – Estonia, FIN – Finland) and date, collector's name and GenBank accession numbers for *28S D1*, *28S D2*, *EF-1α*, *wingless*, *COI*, *GAPDH*, *RpS5* and *IDH* sequences of the studied specimens are indicated. Tribal classification follows Viidalepp (1996) with the exception of using the name Boarmiini instead of Cleorini (following Holloway, 1993). Taxonomy of the generic and species levels was adopted from Müller, 1996.

Species	Collection site and year	Collector	*28S D1*	*28S D2*	*EF-1α*	*wingless*	*COI*	*GAPDH*	*RpS5*	*IDH*
ALSOPHILINAE										
Alsophila aescularia (D. & S. 1775)	EST, Saaremaa, 2002	E. Õunap	GU580695[2]	GU580726[2]	GU580794[2]	GU593332[2]	GU580755[2]	–	–	GU580856[2]
ENNOMINAE										
ABRAXINI										
Abraxas grossulariata (L., 1758)*	EST, Laelatu, 2006	J. Javoiš	HQ340186	HQ340197	HQ340207	HQ340234	HQ340174	HQ340216	–	–
Calospilos sylvata (Scopoli, 1763)	FIN, Hanko, 2004	K .Ruohomäki	GU580704[2]	GU580734[2]	GU580800[2]	GU593336[2]	GU580762[2]	GU580830[2]	GU580668[2]	–
Lomaspilis marginata (L., 1758)	EST, Avinurme, 2005	T. Tammaru	GU580718[2]	GU580748[2]	GU580813[2]	–	GU580777[2]	GU580844[2]	GU580685[2]	GU580877[2]
ANGERONINI										
Angerona prunaria (L., 1758)	EST, Karilatsi, 2005	T. Tammaru	GU580721[2]	–	GU580795[2]	GU593341[2]	GU580756[2]	GU580847[2]	GU580689[2]	GU580881[2]
BISTONINI										
Biston strataria (Hufnagel, 1767)	FIN, Parainen, 2002	H. Takanen	GU580711[2]	GU580741[2]	GU580797[2]	–	GU580759[2]	GU580836[2]	GU580676[2]	GU580872[2]
Biston betularia (L., 1758)	EST, Karilatsi, 2004	T. Tammaru	EF206658[1]	EF206666[1]	EF206673[1]	GU593338[2]	EF206681[1]	GU580832[2]	GU580670[2]	GU580867[2]
BOARMIINI										
Arichanna melanaria (L., 1758)	EST, Karilatsi, 2005	T. Tammaru	GU580714[2]	GU580744[2]	–	–	GU580758[2]	GU580840[2]	GU580680[2]	GU580874[2]
Cleora cinctaria (D. & S. 1775)	FIN, Raisio, 2003	K. Ruohomäki	GU580705[2]	GU580735[2]	GU580801[2]	GU593337[2]	GU580763[2]	GU580831[2]	GU580669[2]	GU580866[2]
Hypomecis punctinalis (Scopoli, 1763)	EST, Avinurme, 2004	T. Tammaru	EF206660[1]	EF206668[1]	EF206675[1]	–	EF206683[1]	GU580825[2]	GU580664[2]	–
BUPALINI										
Bupalus piniaria (L., 1758)	EST, Avinurme, 2004	T. Tammaru	GU580706[2]	GU580736[2]	GU580798[2]	GU593339[2]	GU580760[2]	GU580833[2]	GU580671[2]	–
CABERINI										
Cabera pusaria (L., 1758)	EST, Avinurme, 2004	T. Tammaru	GU580717[2]	GU580747[2]	GU580799[2]	–	GU580761[2]	–	GU580684[2]	GU580876[2]
Lomographa bimaculata (F., 1829)	FIN, Seili, 2003	K. Ruohomäki	GU580697[2]	GU580728[2]	GU580814[2]	–	GU580778[2]	–	–	GU580857[2]
Lomographa temerata (D. & S., 1775)*	EST, Avinurme, 2006	T. Tammaru	HQ340189	HQ340200	HQ340210	HQ340237	HQ340177	–	HQ340228	HQ340220
CAMPAEINI										
Hylaea fasciaria (L., 1758)	EST, Karilatsi, 2005	T. Tammaru	GU580720[2]	GU580750[2]	GU580805[2]	–	GU580768[2]	GU580846[2]	GU580688[2]	GU580880[2]
COLOTOINI										
Colotois pennaria (L., 1761)	FIN, Hanko, 2003	K. Ruohomäki	GU580693[2]	GU580724[2]	GU580802[2]	–	GU580764[2]	–	GU580658[2]	GU580854[2]
ENNOMINI										
Crocallis elinguaria (L., 1758)*	EST, Laelatu, 2006	J. Javoiš	HQ340183	HQ340195	HQ340204	–	HQ340171	HQ340215	–	–
Ennomos fuscantaria (Haworth, 1809)	FIN, Turku, 2000	K. Ruohomäki	EF206659[1]	EF206667[1]	EF206674[1]	GU593335[2]	EF206682[1]	GU580826[2]	GU580665[2]	GU580861[2]
Selenia dentaria (F., 1775)*	EST, Laelatu, 2006	J. Javoiš	HQ340192	HQ340202	HQ340213	HQ340239	–	–	HQ340231	HQ340223
Selenia tetralunaria (Hufnagel, 1767)*	EST, Kurtna, 2006	T. Tammaru	HQ340185	HQ340196	HQ340206	–	HQ340173	–	HQ340226	–
GNOPHINI										
Ematurga atomaria (L., 1758)	EST, Karilatsi, 2005	T. Tammaru	GU580719[2]	GU580749[2]	GU580806[2]	–	GU580769[2]	GU580845[2]	GU580686[2]	GU580878[2]
Siona lineata (Scopoli, 1763)	EST, Karilatsi, 2005	T. Tammaru	EF206663[1]	EF206671[1]	EF206678[1]	GU593340[2]	EF206686[1]	–	GU580687[2]	GU580879[2]
MACARIINI										
Chiasmia clathrata (L., 1758)	EST, Karilatsi, 2005	T. Tammaru	GU580716[2]	GU580746[2]	GU580818[2]	–	GU580788[2]	GU580843[2]	GU580683[2]	GU580875[2]
Itame loricaria (Eversmann, 1837)	EST, Saare, 2004	I. Taal	GU580700[2]	–	GU580811[2]	–	GU580775[2]	–	–	–
Itame brunneata (Thunberg, 1784)	EST, Karilatsi, 2004	T. Tammaru	GU580701[2]	GU580731[2]	GU580810[2]	–	GU580774[2]	GU580824[2]	GU580663[2]	–
Macaria wauaria (L., 1758)*	EST, Laelatu, 2006	J. Javoiš	HQ340184	–	HQ340205	HQ340233	HQ340172	–	HQ340225	HQ340219
Macaria liturata (Clerck, 1759)*	EST, Limnoloogia, 2006	E. Õunap	–	–	–	–	HQ340180	–	–	–

TABLE 1 (continued).

Species	Collection site and year	Collector	*28S D1*	*28S D2*	*EF-1α*	*wingless*	*COI*	*GAPDH*	*RpS5*	*IDH*
Macaria notata (L., 1758)*	EST, Limnoloogia, 2006	E. Õunap	HQ340193	–	–	–	HQ340181	–	–	–
Macaria alternata (D. & S., 1775)*	EST, Karilatsi, 2006	T. Tammaru	HQ340191	–	HQ340212	–	HQ340179	–	HQ340230	HQ340222
OURAPTERYGINI										
Cepphis advenaria (Hübner, 1790)*	EST, Laelatu, 2006	J. Javoiš	HQ340190	HQ340201	HQ340211	HQ340238	HQ340178	–	HQ340229	HQ340221
Opisthograptis luteolata (L., 1758)*	EST, Karuste, 2006	E. Õunap	HQ340194	HQ340203	HQ340214	–	HQ340182	HQ340218	HQ340232	HQ340224
Plagodis pulveraria (L., 1758)*	EST, Avinurme, 2006	T. Tammaru	HQ340188	HQ340199	HQ340209	HQ340236	HQ340176	HQ340217	HQ340227	–
Pseudopanthera macularia (L., 1758)*	EST, Laelatu, 2006	J. Javoiš	HQ340187	HQ340198	HQ340208	HQ340235	HQ340175	–	–	–

* species with gene sequences first determined in the present study; – not available; [1] Viidalepp et al., 2007; [2] Wahlberg et al., 2010.

90% of trees were combined together with LogCombiner v1.5.4. Subsequently, a final tree file was created on the basis of the saved trees using TreeAnnotator v1.5.4 and the results were visualized with FigTree v1.3.1.

In this analysis the 95% credibility intervals for divergence time estimates of each node appeared to be very wide and thus not informative. Therefore, we chose to drop the information on the node ages from the following discussion and concentrated only on the phylogenetic relationships between the studied taxa.

RESULTS AND DISCUSSION

Adding new species to the previously derived phylogenetic tree of Ennominae (Wahlberg et al., 2010) did not result in any changes in the topology of the previously resolved parts of the tree (Fig. 1). In particular, the basal dichotomy into the "ennomine" and "boarmiine" groups of genera, first rigorously shown by Wahlberg et al. (2010), remained valid. No surprise, *Lomographa temerata* and *Abraxas grossulariata* appeared as sisters to *L. bimaculata* and *Calospilos sylvata*, respectively. Similarly, the placement of the three *Macaria* species as sisters to *Itame* was expected (Scoble & Krüger, 2002). Nevertheless, the data presented conflict with the frequently assumed close relationship between *Chiasmia clathrata* and *Macaria* spp. Despite considerable differ-

TABLE 2. Primers and annealing temperatures used in the PCR and cycle sequencing (CS). Universal 5' end tails used for cycle sequencing with primers T7Promoter or T3 are in bold text, if present.

Primer	Primer sequence	Gene region	Direction	PCR	CS	Source
Cov-1f	5'-TCG CTT ATT ATT CAG CCA TTT TAT T-3'	COI, 5' half	Forward	49–52°C	47–52°C	Õunap et al., 2008
Cov-1r	5'-CTG CAC CAT TTT CTA CAA TTC TTC T-3'	COI, 5' half	Reverse	49–52°C	52°C	Õunap et al., 2008
Ron	5'-GGA TCA CCT GAT ATA GCA TTC CC-3'	COI, 3' half	Forward	52°C	50°C	Caterino & Sperling, 1999
Nan	5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3'	COI, 3' half	Reverse	50–52°C	47–50°C	Õunap et al., 2005
D1F	5'-GGG GAG GAA AAG AAA CTA AC-3'	28S D1	Forward	50–60°C	47–55°C	Abraham et al., 2001
D1R	5'-CAA CTT TCC CTT ACG GTA CT-3'	28S D1	Reverse	50–60°C	47–55°C	Abraham et al., 2001
D2F	5'-AGA GAG AGT TCA AGA GTA CGT G-3'	28S D2	Forward	55–61°C	55°C	Belshaw & Quicke, 1997
D2R	5'-TTG GTC CGT GTT TCA AGA CGG G-3'	28S D2	Reverse	55–61°C	55°C	Belshaw & Quicke, 1997
LepWG1	5'-GAR TGY AAR TGY CAY GGY ATG TCT GG-3'	Wingless	Forward	58–63°C	57–58°C	Brower & DeSalle, 1998
LepWG3	5'-ACT YCG CAR CAC CAR TGG AAT GTR CA-3'	Wingless	Reverse	58–63°C	57–58°C	Brower & DeSalle, 1998
ef44	5'-GCY GAR CGY CAR CGT GGT ATY AC-3'	EF-1α	Forward	58–60°C	55–58°C	Monteiro & Pierce, 2001
efrcM4	5'-ACA GCV ACK GTY TGY CTC ATR TC-3'	EF-1α	Reverse	58–60°C	55–58°C	Monteiro & Pierce, 2001
Cho2	5'-CTA CGT CAC CAT CAT CGA-3'	EF-1α, 5' half	Forward	58°C	57°C	Viidalepp et al., 2007
Verdi4	5'-CAC CAG TCT CCA CAC GGC C-3'	EF-1α, 5' half	Reverse	58°C	57°C	Viidalepp et al., 2007
EF51.9	5'-CAR GAC GTA TAC AAA ATC GG-3'	EF-1α, 3' half	Forward	58°C	57°C	Monteiro & Pierce, 2001
Niina2	5'-CCT GGA AGG ACT CCA CRC ACA G-3'	EF-1α, 3' half	Reverse	58°C	57°C	Viidalepp et al., 2007
HybFrigga	5'-**TAA TAC GAC TCA CTA TAG GG**A ARG CTG GRG CTG AAT ATG T-3'	GAPDH	Forward	55°C	–	Wahlberg & Wheat, 2008
HybBurre	5'-**ATT AAC CCT CAC TAA AG**G WTT GAA TGT ACT TGA TRA GRT C-3'	GAPDH	Reverse	55°C	–	Wahlberg & Wheat, 2008
HybrpS5degF	5'-**TAA TAC GAC TCA CTA TAG GG**A TGG CNG ARG ARA AYT GGA AYG A 3'	RpS5	Forward	55°C	–	Wahlberg & Wheat, 2008
HybrpS5degR	5'-**ATT AAC CCT CAC TAA AG**C GGT TRG AYT TRG CAA CAC G 3'	RpS5	Reverse	55°C	–	Wahlberg & Wheat, 2008
IDHdeg27F	5'-**TAA TAC GAC TCA CTA TAG GG**G GWG AYG ARA TGA CNA GRA THA THT GG-3'	IDH	Forward	55°C	–	Wahlberg & Wheat, 2008
IDHdegR	5'-**ATT AAC CCT CAC TAA AG**T TYT TRC AIG CCC ANA CRA ANC CNC C-3'	IDH	Reverse	55°C	–	Wahlberg & Wheat, 2008
T7Promoter(F)	5'-TAA TAC GAC TCA CTA TAG GG-3'	GAPDH, RpS5, IDH	Forward	–	45°C	Regier & Shi, 2005
T3(R)	5'-ATT AAC CCT CAC TAA AG-3'	GAPDH, RpS5, IDH	Reverse	–	45°C	Wahlberg & Wheat, 2008

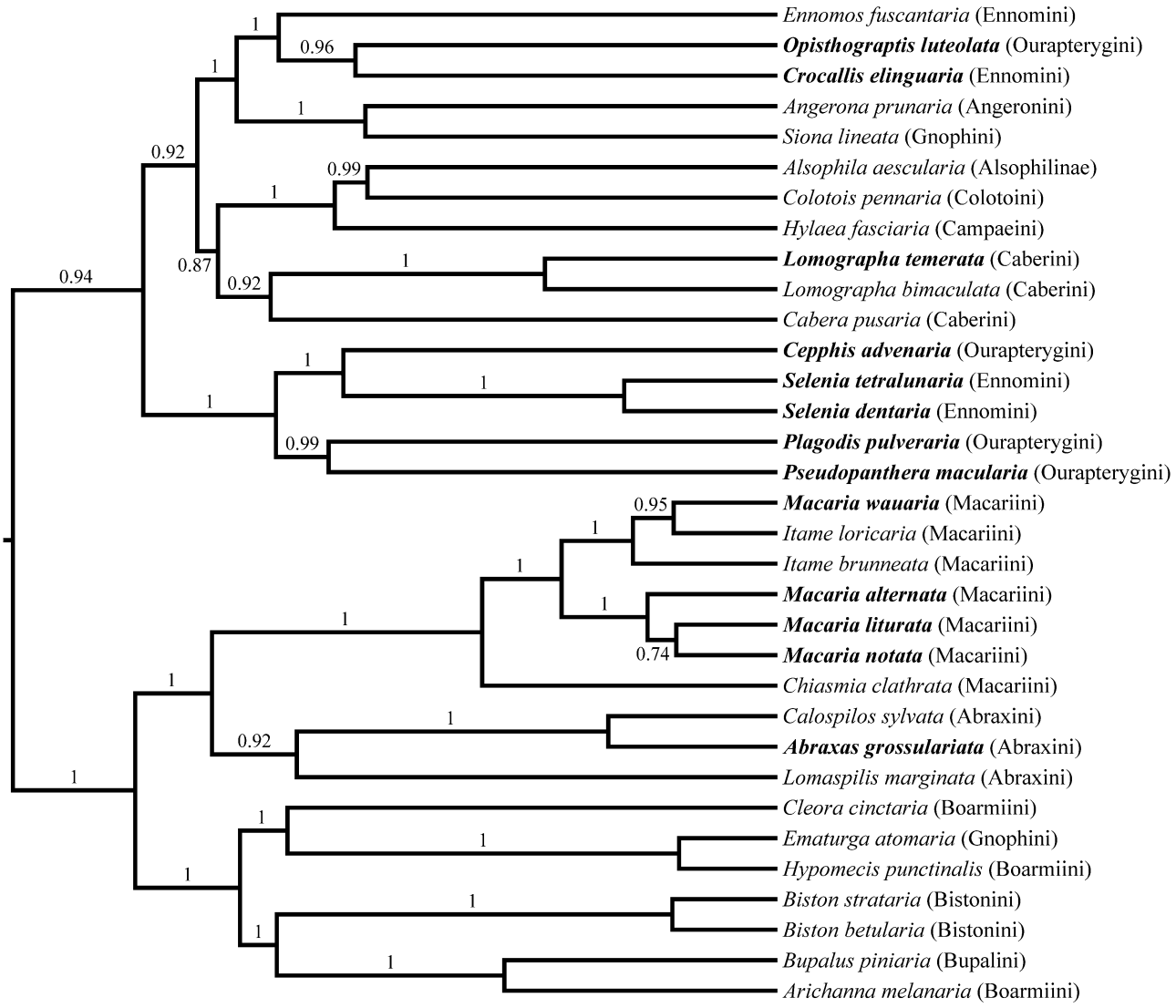

Fig. 1. Bayesian phylogenetic tree (GTR + I + Γ model) of selected European Ennominae based on a 4718 bp combined sequence of *COI*, *28S*, *EF-1α*, *wgl*, *GAPDH*, *IDH* and *RpS5* sequences. The numbers above or below branches are Bayesian posterior probabilities. The original contribution is highlighted: the species first examined using molecular methods in the current study are indicated in bold.

ences in the appearance and ecology of these moths, they are frequently treated as congeneric in European literature (though not so in the most recent publications: Müller, 1996, Scoble & Krüger, 2002), which plausibly follows Wehrli's (1939–1953) combining *Chiasmia* with *Semiothisa* as a subgenus. Moreover, the data presented here show that the recent suggestion to transfer *wauaria* from *Itame* (or *Speranza*, following Ferguson, 2008) to *Macaria* (e.g. Müller, 1996) is not justified, as it would make both *Itame* and *Macaria* paraphyletic.

In the "ennomine" branch, the genera *Cepphis, Plagodis, Pseudopanthera* and *Selenia* form a well supported monophyletic entity, which does not include any of the species from Wahlberg et al.'s (2010) data set. Close relationships between these genera is not surprising because all were included in the tribe Ennomini by Herbulot (1961–1962). On the other hand, this study indicates that *Ennomos* is not closely related to the four above mentioned genera, but forms a common clade with *Crocallis* and *Opisthograptis*. The two latter genera are, however, also placed in Ennomini by Herbulot (1961–1962) but the phylogenetic tree revealed by this study shows that Ennomini sensu Herbulot is not justified as it would be paraphyletic with respect to several other currently recognised tribes (Fig. 1). Moreover, the way Viidalepp (1996) divides these genera between Ennomini and Ourapterygini (Table 1) is also not supported. In contrast, the clade consisting of *Cepphis, Plagodis, Pseudopanthera* and *Selenia* matches the concept of Anagogini of Forbes (1948) – subsumed to Hypochrosini by Holloway (1993) – pointing to the conclusion that the idea of "reviving" this tribe should deserve more attention from taxonomists.

In the "ennomine" branch of the subfamily, the present analysis revealed 4 to 6 monophyletic groups, which could be considered to represent different tribes. Even if these clades received reasonably high support and the

topology presented here does not conflict with Wahlberg et al. (2010), the currently available studies include only a fraction of the total diversity of Ennominae. Any suggestions for taxonomic rearrangements are therefore clearly premature. Nevertheless, phylogenetic relationships among a sufficient number of north European Ennominae are now known well enough to facilitate using this information in comparative studies. Notably, body size appears to be an evolutionarily plastic trait in this group. For instance, the similarly looking stout-bodied moths in the genera *Colotois*, *Selenia*, *Ennomos* and *Crocallis* are not closely related to each other, but all have small and slender-bodied relatives (*Opisthograptis*, *Alsophila*, *Cephis*). Multiple independent evolutionary changes in body size should create a favourable situation for studies on morphological and ecological correlates of this important life history trait.

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