Exploring phenotypic plasticity and biogeography in emerald moths: A phylogeny of the genus Nemoria (Lepidoptera: Geometridae)

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ARTICLE INFO

Article history:
Received 21 November 2007
Revised 27 June 2008
Accepted 4 July 2008
Available online 15 July 2008

Keywords:
Lepidoptera
Geometridae
Geometrinae
Nemoria
Phenotypic plasticity
Polyphenism
Larvae
Caterpillar

ABSTRACT

The moth genus Nemoria (Lepidoptera: Geometridae) includes 134 described species whose larvae and adults display a considerable range of phenotypic plasticity in coloration and morphology. We reconstructed the phylogeny of 54 species of Nemoria and seven outgroups using characters from the mitochondrial genes, Cytochrome Oxidase I and II (COI and COII), and the nuclear gene, Elongation Factor-α (EF-1α). Maximum parsimony, maximum likelihood and Bayesian inference were used to infer the phylogeny. The 54 ingroup species represented 13 of the 15 recognized species groups of Nemoria [Ferguson, D.C., 1985. Fasc. 18.1, Geometridae: Geometridae (in part). In: Dominick, R.B. (Ed.), The Moths of America North of Mexico, Fasc. 18.1. Wedge Entomological Research Foundation, Washington; Pitkin, L.M., 1993. Neotropical emerald moths of the genera Nemoria, Liosochlora and Chavarriella, with particular reference to the species of Costa Rica (Lepidoptera: Geometridae, Geometrinae). Bull. Br. Mus. Nat. Hist. 62, 39–159], and the seven outgroups came from four tribes of Geometrinae. These data support Nemoria as a monophyletic group and largely recover the species groupings proposed in previous taxonomic analyses using morphological characters. Phenotypic plasticity of larvae is not correlated with plasticity of adults among those species of Nemoria where life histories are known, and appears to be evolutionarily labile for both life history stages; Species exhibiting larval phenotypic plasticity, such as N. arizonaria and N. outina, are placed in several distinct clades, suggesting that this trait has evolved multiple times, and species displaying adult phenotypic plasticity are likewise distributed throughout the phylogeny. A comparative analysis of the biogeographic history of Nemoria supports a South American origin for the genus with multiple introductions into North America, and an application of published substitution rates to the phylogram provides an age estimate of 7.5 million years.

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1. Introduction

The ability of organisms to produce alternative phenotypes in response to environmental cues has led to the evolution of phenomena such as caste formation in social insects, temperature-dependent sex determination in fish and turtles, and seasonal plasticity in many moths and butterflies. Considerable progress has been made in understanding some of the underlying mechanisms giving rise to environmentally induced variation, or phenotypic plasticity (e.g. Kucharski et al., 2008; Suzuki and Nijhout, 2008), but less attention has been paid to the evolution of these traits in specific groups. A phylogenetic approach can provide additional perspectives on how and why phenotypic plasticity evolves. In this study, we investigated the evolution of a group of geometrid moths that display remarkable diversity in both larval and adult forms.

The moth family Geometridae contains over 21,000 species with a worldwide distribution (Scoble, 1999) and has been the focus of a number of recent systematic studies (Abraham et al., 2001; Yamamoto and Sota, 2007). Several studies have also explored its wealth of diversity in morphology and behavior (e.g. Akino et al., 2004; Brehm et al., 2005; Brehm and Sullivan, 2005; Mutanen and Kaitala, 2006). The subfamily Geometrinae contains approximately 2350 species (Hausmann, 2001), many of which are characterized by the emerald green pigmentation from which their common name, the emerald moths, is derived. Variation in morphology of both larvae and adults is a striking characteristic of many of the species of this group, and study of their systematic relationships and biogeographic history provides an opportunity to make a comparative analysis of the evolution of phenotypic plasticity.

Nemoria is the largest genus of New World Geometrinae. The genus contains 134 described species that are distributed throughout southern Canada, the United States, Central America and the
Caribbean to South America. In contrast to other closely related geometrine moths, the distributions of Nearctic Nemoria species are often quite restricted (Ferguson, 1985). The Neotropical species also seem to be more localized than other geometrid genera (Pitkin, 1993). The adults typically exhibit emerald green wing patterns with small patches of red and black. Larvae feed on a wide range of host plants, from woody plants to herbaceous foliage and flowers, and although host plant affiliations have been determined for some species (Ferguson, 1985; Wagner, 2005), the immature stages of most species remain unknown. The majority of the diversity in this genus can be found in the tropical regions of Central America, but significant diversity also exists in the oak highlands of the southwestern United States and Mexico.

Nemoria has been the subject of two taxonomic reviews. Ferguson (1985) revised the taxonomy of Nemoria and several other genera of North American Geometrinae, and included extensive notes on distributions and ecology. A second revision (Pitkin, 1993) focused on the Nemoria species in Costa Rica, about which much less is known ecologically. Together, these two treatments divided the 134 species into eight Nearctic species groups and seven Neotropical groups (see Table 1). Nemoria species are characterized by green wing patterns and complex and diverse male genitalia characters, including a distinctive basal costal process that, when present, is highly characteristic of this genus (Ferguson, 1985).

Nemoria is most widely known because of the phenotypic plasticity exhibited by larvae of some species. For example, the larvae of Nemoria arizonaria become catkin mimics when they feed on oak catkins in the spring, but twig mimics when they consume oak leaves in the summer (McFarland, 1988; Greene, 1989). Another species, N. outina, also has two distinctly different forms (Deyrup and Eisner, 1993) as the result of eating leaves of different ages. The adults typically exhibit emerald green wing patterns, and Eisner, 1993) as the result of eating leaves of different ages. For example, the larvae of Nemoria, also exhibit distinctive, variable phenotypes that are determined by environmental cues.

In Nemoria, eight species display adult wing pattern variation that is the likely result of phenotypic plasticity. Four of these species (N. bistriaria, N. bifilata, N. efla and N. viridicaria) have both a green form characteristic of this genus, and a brown form in which the wings are reddish-brown (Buckett and Sears, 1968; Ferguson, 1985). In all of these species, the green form appears in the summer, and the brown form appears in the spring. The brown form of another species, N. pulcherrima, appears along with the green form and does not display a pattern typical of phenotypic plasticity cued by seasons. Two species, N. lixaria and N. efla, have a semi-melanic winter form where blackened scales are produced on winter forms, and while they also exhibit naturally occurring phenotypic plasticity, the presentation and underlying basis of the color differences are significantly different and suggest that different pigments are involved. Ferguson (1985) proposed that two species from the southwestern United States of America (USA), N. arizonaria and N. daedalea are closely related species, and they also have seasonal variation in wing coloration. In these two species, the late winter-early spring brood has more distinct green coloration and a dark purplish-red costa, where the summer form is paler with less distinct abdominal markings. These forms are more distinct in N. arizonaria than in N. daedalea, and the N. arizonaria forms have such striking differences that they were originally described as different species (Ferguson, 1985).

In the study presented here, we infer the phylogeny of 54 Nemoria species using molecular characters from two mitochondrial genes, Cytochrome Oxidase I and II (COI and COII), and one nuclear gene, Elongation Factor-1α (EF-1α). These genes were chosen because they have been successful in resolving phylogenetic relationships in other studies of lepidopteran systematics focusing on species relationships within genera of comparable ages (Monteiro and Pierce, 2001; Rand et al., 2000; Kandul et al., 2004; Zakharov et al., 2004). Our results provide the first formal hypothesis of the phylogenetic relationships within Nemoria, explore the biogeographic history of the group and give a glimpse as to how larval and adult phenotypic plasticity may have evolved in the group.

2. Materials and methods

2.1. Taxon sampling and choice of outgroup

The 63 specimens used in this study represent 54 species of Nemoria, and include exemplars of seven of the eight species groups identified by Ferguson in his 1985 revision, and six of the seven groups proposed by Pitkin in her 1993 study (Table 1; more detailed collection data available from MRC). The tribal classification of the Geometrinae remains uncertain (Pitkin, 1996), and the seven species selected as outgroups represent four tribes: Nemorini, Synchlorini, Lophochoristini and Hemitheni.

2.2. DNA isolation

Most of the sample specimens were collected in the field and killed by freezing or pinching the thorax of the adult. These were then preserved in 95% ethanol and kept at −80°C. For some of the Neotropical Nemoria species that were difficult to collect, DNA was extracted from dried specimens loaned from the Instituto Nacional de Biodiversidad (INBio) in Costa Rica. Whole genomic DNA was isolated by grinding 1–2 legs and sometimes segments 1 and 2 of the abdomen in lysis buffer, followed by purification using the DNeasy Tissue Kit (Qiagen, Valencia, CA) following the instructions of the manufacturer.

2.3. Amplification using PCR

Gene fragments for COI, COII and EF-1α were amplified using PCR (Mullis et al., 1986; Saiki et al., 1988). Published primers were used in most cases, but for some of the dried specimens, specifically designed internal primers (available upon request) were used to amplify smaller fragments (150–250 bp) (Table 2). COI fragments were amplified for all taxa (63 taxa), whereas COII and EF-1α were only successfully amplified for those specimens that had been collected into alcohol (41 and 39 taxa, respectively). PCR was completed on a DNA Engine thermal cycler (MJ Research PTC-200) using the following reagents (with some modifications in particular reactions): 25 μL reactions of 2.5 μL Q solution, 2.5 μL buffer, 2 μL MgCl₂, 0.25 μL Taq (from the Qiagen Taq DNA Polymerase kit), 13.5 μL water, 0.25 μL dNTPs, 1.25 μL of each primer and 1.5 μL DNA. Reactions for COI and COII amplification began by denaturing at 95°C for 2 min, followed by 37 cycles in a touchdown PCR program starting at 48°C. A similar program was used that started at an annealing temperature of 53°C for EF-1α. Adjustments to these profiles were made for fragments that did not amplify easily. PCR product was cleaned either using QIAquick PCR purification kits (Qiagen) or a 4:1 mixture of shrimp alkaline phosphatase (Roche Applied Science, Indianapolis, IN #1758250) and exonuclease I (Fermentas USA, Hanover, MD #EN0581).

2.4. Sequencing and alignment

Sequencing reactions for all fragments in this study were completed using ABI Prism 2 dye terminator cycle sequence
Table 1
List of all specimens, species group, geographic range (after Ferguson, 1985 and Pitkin, 1993), collection numbers, and GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Geographic range</th>
<th>Collection #</th>
<th>COI Acc. #</th>
<th>COII Acc. #</th>
<th>EF-1α Acc. #</th>
</tr>
</thead>
</table>

Abbreviated directions: N, north; S, south; W, west; E, east. Countries: USA, United States of America; CAN, Canada; MEX, Mexico. States in the USA: AL, Alabama; AZ, Arizona; CA, California; CO, Colorado; FL, Florida; GA, Georgia; KA, Kansas; KY, Kentucky; LA, Louisiana; MD, Maryland; MO, Missouri; MS, Mississippi; ND, North Dakota; NV, Nevada; NJ, New Jersey; NM, New Mexico; NC, North Carolina; OR, Oregon; SC, South Carolina; SD, South Dakota; TN, Tennessee; TX, Texas; UT, Utah; VA, Virginia; WA, Washington; WV, West Virginia.

on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequences were aligned using Sequencer 3.1.1 (Genecodes, Ann Arbor, MI) in comparison with total mtDNA sequence (GenBank No. NC_002355, Lee and Kim, 1999) and EF-1x sequence (GenBank No. D13338, Kamiie et al., 1993) of Bombyx mori L. (as in Kandul et al., 2004). All alignments in the study were unambiguous for both the mitochondrial and nuclear genes. Primer sequences were removed from these fragments and the final alignment was completed in MacClade (Maddison and Maddison, 2003).

2.5. Phylogenetic analysis

For phylogenetic analyses, sequences from the mitochondrial genes COI and COII (63 taxa) and then the nuclear gene EF-1x (39 taxa) were analyzed separately and then all data from COI, COII and EF-1x (63 taxa) were concatenated using MacClade (Maddison and Maddison, 2003) and analyzed as a combined dataset. A variety of optimality criteria were used. Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference were used to infer relationships among taxa (Felsenstein, 1978; Hasegawa and Fuwara, 1993; Kuhner and Felsenstein, 1994; Huelsenbeck, 1995).

 Parsimony heuristic searches in PAUP* 4.0b10 (Swoford, 2001) using equal character weighting consisted of 1000 replicates with random stepwise addition, branch bisection-reconnection (TBR) branch swapping, collapsing zero-length branches. Three different parsimony searches were performed on the data. We analyzed each of the following data sets in separate searches: the COI and COII fragments combined (analyzed together since the mitochondria acts as a single locus); the EF-1x nuclear fragment; and the total combined data set. When a particular search produced more than a single most parsimonious tree, a strict consensus of the trees was made.

To determine the most appropriate substitution model for the maximum likelihood (ML) analyses, we used the likelihood ratio test (LRT) in Modeltest 3.06 (Posada and Crandall, 1998). A heuristic search was implemented in the software package PhyML v. 2.4 (Guindon and Gascuel, 2003) to repetitively search for trees until the process converged on a single tree. For bootstrap (bs) searches 500 pseudoreplicates of the dataset (Felsenstein, 1985; Hillis and Bull, 1993) were used to measure the support of nodes on the parsimony and maximum likelihood trees using the closest stepwise addition of the heuristic search.

Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). Model parameters were estimated during each run using Markov chain Monte Carlo sampling with a default heating value of 0.2. The Bayesian analyses were run for 10,000,000 generations with trees sampled every 1000 generations after an initial burn in period of 10,000 generations. Bayesian posterior probabilities (bpp) were then estimated using the majority rule consensus trees created in PAUP* 4.0b10. The default setting in MrBayes 3.1.1 runs two simultaneous independent analyses, computing the standard deviation of the split frequencies between the two analyses at regular intervals as a means of assessing convergence of the two independent runs.

2.6. Age estimation of the genus Nemoria

We tested for the significance of a molecular clock using the LRT with and without a molecular clock enforced. Because the LRT found significant deviations from rate constancy (i.e. non-clock-like) (P < 0.001), we used mean uncorrected pairwise distances, calculated in MEGA 2.1 (Kumar et al., 2001) using only the data for the mitochondrial gene COI, since this is the only gene for which we had all taxa sequenced, and estimates for the rate of evolution for this locus in insects are available (see citations below). Standard error was estimated by bootstrap method with 10,000 replications and random number seed. Published estimates of substitution rates were applied to the recovered phylogram. Within Insecta, the substitution rate for COI converges on approximately 1.5% pairwise distance per million years (Brower, 1994; Schuhart et al., 1998; Quek et al., 2004). We therefore applied this substitution rate to date main phylogenetic events on the recovered phylogeny.

2.7. Ancestral character state reconstruction

The program Mesquite v. 1.12 (Maddison and Maddison, 2006) was used in ancestral character state analyses. Both ML and MP ancestral character reconstruction methods were used with our maximum likelihood phylogeny. Maximum likelihood optimizations used the Markov k-state one-parameter model (Lewis, 2001). Characters considered were adult phenotypic plasticity.
and juvenile phenotypic plasticity. For each, character states were coded as 0 = phenotypic plasticity absent and 1 = phenotypic plasticity present. We coded adult phenotypic plasticity simply for presence or absence; our sample size was not large enough to make a more detailed breakdown of different forms of adult plasticity, such as seasonal green and brown forms versus semi-melanic winter forms (Ferguson, 1985). For the purpose of the larval analysis, phenotypic plasticity was considered absent as a default.

2.8. Historical biogeography

The biogeographic program LAGRANGE 1.0.1 (Ree et al., 2005) was used to infer the historical geographic range on the Nemoria phylogeny. LAGRANGE is a python package that uses likelihood-based methods to perform historical biogeographic analyses. The procedure used in LAGRANGE differs from previous dispersal-vicariance analysis methods (DIVA; Ronquist 1996, 1997) in that it allows a broader range of speciation models and also incorporates any available temporal information such as divergence times and dispersal opportunities. Global likelihood calculations are based on these inheritance scenarios and transition probabilities estimated using Monte Carlo methods.

The model used is similar to that used in the Cercis empirical example in Ree et al. (2005). Since LAGRANGE 1.0.1 requires the root-to-tip length in millions of years and the only available age estimate was that of the genus Nemoria itself, for this biogeographic analysis the eight outgroups in other genera were omitted from the ML topology. Also, as LAGRANGE 1.0.1 is only capable of handling ultrametric trees, branches were ultrametricized in Mesquite (Maddison and Maddison, 2006). The inferred age estimate was used as the root-to-tip length for the Nemoria only tree. Nemoria species are distributed in the Neotropics and either western or eastern North America, and for the analysis, each taxon was designated as belonging to one of three areas (EN—eastern North America, WN—western North America and CS—Neotropical Central and South America); the run times increase exponentially with the number of areas in the model, and the large number of internodes in the Nemoria phylogeny already makes analyses slow since run time increases linearly with internode number (Ree et al., 2005).

Connections between the three areas were parameterized as follows: as the all areas were always interconnected by land in the time period under consideration, the probability of dispersal success was assumed to be one over the time period under consideration (in this case 7.5 million years ago) between each area (i.e. between EN and WN, between EN and CS and between WN and CS). This connection parameter corresponds to the within continent probability of dispersal success (e.g. Ree et al., 2005).

Inferences were made over a range of parameter values for lineage dispersal and extinction. The rate of dispersal to other areas ($\lambda_D$) and the rate of extinction within an area ($\lambda_E$) are assumed to be constant across lineages and through time. As there is no information on dispersal and extinction rates, three scenarios were considered for both “high” rates ($\lambda_D > \lambda_E = 0.1$, which corresponds to an average of one event per 10 million years) and “low” rates ($\lambda_D > \lambda_E = 0.01$, or one event per 100 million years): $\lambda_D > \lambda_E$, $\lambda_D = \lambda_E$ and $\lambda_D < \lambda_E$ (e.g. Ree et al., 2005). To obtain better estimates, 100,000 iterations were run for each starting area in calculating the range transition matrix ($P$) for each branch.

3. Results

3.1. Sequence statistics

The COI and COII combined fragment was 2165 bp with 206 variable and 639 (29.5%) parsimony informative characters. The combined analysis of all three genes (COI, COII and EF-1α) provided fragments of approximately 3151 bp. For the complete dataset, 257 were variable and 857 (27%) were parsimony informative. The base composition for the combined data set is: A = 0.31088; C = 0.16516; G = 0.16127; T = 0.36269.

3.2. Maximum parsimony analysis

In the COI and COII analysis, the heuristic search resulted in one most parsimonious tree ($L = 3978$) with a consistency index (CI) of 0.313 and retention index (RI) of 0.398. In the EF-1α analysis, the heuristic search recovered 3721 most parsimonious trees ($L = 736$) with CI = 0.527 and RI = 0.691. Heuristic searches of the combined data set resulted in five equally parsimonious trees ($L = 4674$) and the strict consensus of these trees had a CI of 0.341 and RI of 0.451 (Fig. 1). The overall topologies recovered from the individual gene analyses did not differ from the combined data set (results not shown).

3.3. Likelihood analyses

The LRT implemented in Modeltest selected the GTR+Γ model for all data sets. For the COI and COII data set, the likelihood tree resulting from the analysis had a likelihood score of $-\ln L = 20330.55611$. The GTR relative rate parameters were: $\ln \kappa = 7.75717$; $\ln \kappa = 22.55089$; $\ln \kappa = 3.02547$; $\ln \kappa = 5.46392$; $\ln \kappa = 92.73226$; $\ln \kappa = 1.0$ and base composition was $A = 0.39329$; $C = 0.09238$; $G = 0.08654$; $T = 0.42868$, with $\alpha = 0.387$. The EF-1α tree had a score of $-\ln L = 5054.85199$ and the GTR relative rate parameters were: $\ln \kappa = 1.98196$; $\ln \kappa = 7.00989$; $\ln \kappa = 4.11520$; $\ln \kappa = 1.01496$; $\ln \kappa = 20.05116$; $\ln \kappa = 1.0$ and base composition was $A = 0.25596$; $C = 0.25715$; $G = 0.23267$; $T = 0.25422$, with $\alpha = 0.163$. Finally, the tree resulting from the combined data set (Fig. 2) had a score of $-\ln L = 25556.46576$, and the GTR relative rate parameters were: $\ln \kappa = 4.93891$; $\ln \kappa = 24.80498$; $\ln \kappa = 10.89298$; $\ln \kappa = 3.69035$; $\ln \kappa = 51.72075$; $\ln \kappa = 1.0$ and base composition was $A = 0.29780$; $C = 0.16942$; $G = 0.15870$; $T = 0.37408$, with $\alpha = 0.221$. Again, the overall topologies recovered from the individual gene analyses did not differ from the combined data set (results not shown).

3.4. Bayesian analyses

The Bayesian analysis of the combined data set with a GTR+Γ model of sequence evolution resulted in a tree (Fig. 3) with a likelihood score of $-\ln L = 25578.70498$. The GTR relative rate parameters were: $\ln \kappa = 5.37499$; $\ln \kappa = 26.87757$; $\ln \kappa = 11.55195$; $\ln \kappa = 3.78612$; $\ln \kappa = 59.89581$; $\ln \kappa = 1.0$ and base composition was $A = 0.302155$; $C = 0.162981$; $G = 0.157554$; $T = 0.377309$, with $\alpha = 0.221$. Convergence of the two simultaneous independent analyses implemented in MrBayes 3.1.1, was met with an average standard deviation of split frequencies of 0.007032.

3.5. Dating nodes on the tree

To calibrate a molecular clock, one or more calibration points must be linked to a particular geological or phylogeographic event to permit scaling of rates and times to absolute times. Unfortunately, the present data are not amenable to such an approach. Only two significant known fossils are known for Geometroidea (Grimaldi and Engel, 2005), and neither belong to the groups included in this study. Using MEGA 2.1, we calculated the mean uncorrected pairwise distances for the Nemoria clade on the phylogeny, which was recovered in all analyses. We used a divergence rate for COI of 1.5% per million years (Brower, 1994; Schubart et al., 1998).
1998; Quek et al., 2004). The node containing all members of Nemoria represents 11.2% mean divergence between its descendent sister taxa, which corresponds to an age of 7.5 ± 0.06 million years using the COI divergence rate estimate of 1.5% per million years.

3.6. Phylogenetic relationships within Nemoria

Nemoria was topologically recovered as a monophyletic group in all of our analyses (Fig. 3). In the MP and ML analyses of the combined dataset, this group has moderate bootstrap support (56% and 51%, respectively), while in the Bayesian analysis, it has strong support with a posterior probability (bpp) value of 95%. Although some parts of the tree lack resolution, a number of important groups are supported. First, the southeastern taxa N. elfa + N. tuscarora + N. cataclia + N. outina had 100% bootstrap support in both the MP and ML analyses, and a bpp of 100% in the Bayesian analysis. Several Neotropical groups (N. acutularia + N. remotia + N. dentilinnea + N. defectiva + N. florae, bpp = 95%; N. tufala + N. pacificaria + N. caroliniae, bpp = 100%, MP bs = 99%, ML bs = 100%) also had strong support. However, species with different geographic ranges were also recovered in well supported clades, including the Neotropical taxa, N. karlae, N. rectiliea and N. loranae, which were grouped in the same clade as N. leptalea and N. glaucomarginaria from the western USA (bpp = 100%, MP bs = 72%, ML bs = 79%). Species with both western (N. festaria and N. caerulescens) and eastern distributions in the USA (N. rubfrinaria, N. bistra,ria, N. mimosaria, N. satirubia, N. lixaria and N. bifilata) also clustered in a moderately supported monophyletic group (bpp = 99%, ML bs = 58%, Fig. 3).

3.7. Ancestral state reconstruction

Parsimony-based ancestral character state reconstruction suggests that in this phylogeny, adult phenotypic plasticity has five steps, and juvenile phenotypic plasticity six steps. Maximum likelihood results are similar and are shown in Fig. 3. Log likelihood of the adult phenotypic plasticity character reconstruction is −24.57, and for the juvenile phenotypic plasticity character reconstruction is −28.36. Both methods show multiple independent origins of phenotypic plasticity in Nemoria.
3.8. Historical biogeography

Ancestral geographic ranges were inferred using a likelihood framework in LAGRANGE 1.0.1. The highest overall likelihood (\(\ln L = -289.646\)) was obtained at a high rate of dispersal and extinction in which \(\lambda_D > \lambda_E\) (\(\lambda_D = 0.09, \lambda_E = 0.01\)). From these analyses, it appears Nemoria originated in Neotropical Central and South America and radiated into North America in seven separate colonization events over time (Fig. 4). Colonization of North America happened at nodes 2, 6, 7, 8, 9, 11 and 12. All but one of these events included a radiation into western North America. The most widespread ancestral range occurred at nodes 5 and 6. At node 5, the eastern North American lineage most likely diverged from the rest. Similar speciation scenarios in which a more widespread ancestral population splits into two lineages also occur at nodes 3, 4 and 10. Results suggest that at node 4, a speciation event happened in the Neotropics with no reduction in geographic range for the remaining ancestral species, and this southern species then radiated once again into western North America at node 2. At node 1, the ancestral population appears to have spread throughout North America and subsequently diverged into eastern and western lineages.

4. Discussion

4.1. Phylogenetic relationships within Nemoria

The species of Nemoria are distributed from Canadian provinces to South America, utilize a diverse array of host plants and comprise the largest genus in Geometrinae. This study tentatively supports the monophyly of Nemoria, but a more comprehensive sample would be needed to test this properly. The Bayesian topology of Fig. 3 agrees with many of the taxonomic groupings of Ferguson (1985) and Pitkin (1993) and suggests a congruence of genital and morphological characters in establishing natural groups. Ferguson's (1985) species groups I, III and IV correspond to monophyletic groups recovered in this study (Fig. 3). Represen-
tatives of group II (N. arizonaria, N. viridicaria and N. albaria) show strong support in all phylogenetic analyses, but N. diamesa is placed with relatively low support in a different clade. In the hypothesis presented here, groups VI and VIII are paraphyletic, and two of these species from group VIII (N. leptalea and N. glaucomarginaria) are contained in a monophyletic group with three species from Pitkin’s (1993) cosmeta group. The relationships within this clade suggest that N. leptalea and N. glaucomarginaria should be considered members of the cosmeta group. The remaining representatives of group VIII and those of group V (N. lixaria and N. saturiba) also form a clade.

Of the Neotropical species groups suggested by Pitkin (1993), the pacifica, pulveraria and strigaria groups are supported as monophyletic. The phylogenetic hypothesis presented here also suggests that the erina and scriptaria groups are polyphyletic. This hypothesis also indicates that several other Neotropical species can be tentatively assigned to Pitkin’s groups. Nemoria rectilinea was placed with N. karlae (100% support in all analyses), suggesting that it belongs in the cosmeta group. The presence of N. carolinae in a clade with N. tutala and N. pacifica (bpp = 100%, MP bs = 99%, ML bs = 100%) indicates that N. carolinae belongs in the pacifica group. Finally, five species that Pitkin did not place in any group resolved as a clade in the Bayesian analysis (N. acutularia, N. remota, N. dentilinea, N. defectiva and N. florae; 95% bpp) and we propose that they belong to a new group tentatively assigned as the “acutularia” group.

Fig. 3. Bayesian inference tree (10,000,000 generations) of the combined COI + COII + EF-1α data set using a GTR+Γ model of sequence evolution. Posterior probabilities are shown above nodes. Bootstrap values of nodes supported in both the maximum parsimony (below nodes) and maximum likelihood analysis (below nodes in parenthesis) are also shown.
4.2. Biogeography and age of Nemoria

This genus is most diverse in the Neotropics, with approximately three quarters of the described taxa residing there. One hypothesis for the historical biogeography of this group is that Nemoria species originated in the tropics and radiated into North America. Many of the Neotropical species are recovered in the basal portion of the trees in this study, a result that is consistent with this hypothesis. However, the data presented here do not support a single colonization of North America but rather multiple independent colonizations. The mix of Neotropical and Nearctic taxa in many of the clades support this view of the geographic radiation of Nemoria. For example, the Neotropical taxa N. karlai, N. rectilinea and N. loranae were contained in the same group as N. leptalea and N. glaucomarginaria from the western United States.

The geographic ranges of the Nearctic Nemoria species are generally concentrated in the western and southwestern North America and in the eastern and southeastern areas of North America. The results of this study support the relationships in Ferguson’s (1985) species groups and suggest that the eastern and western species of Nemoria do not form two distinct clades. The southeastern U.S. clade of N. efla, N. tuscatora, N. catachloa and N. outina is not sister to the other species distributed throughout the eastern USA such as N. bistriaria, N. bifilata and N. mimosa. These eastern species instead are more closely related to species such as N. festaria and N. caerulescens found in the western USA. Although a more comprehensive sampling of species will allow for a more thorough biogeographic analysis, this study argues against a simple pattern of geographic division among the major Nemoria clades.

It is not surprising to find that this preliminary biogeographic analysis suggests that the majority of the radiation events were colonizations of western North America as opposed to eastern North America. Western North America is directly connected to Central America, and colonizations of eastern North America...
would involve either migrating through Texas or crossing the Gulf of Mexico (which is bounded on the northeast, north and northwest by the Gulf Coast of the United States, on the southwest and south by Mexico, and on the southeast by Cuba). This analysis assumed equal dispersal times from the Neotropics to eastern and western North America; dispersal time to eastern North America may be a bit longer.

The biogeographic results of this study support previous hypotheses that Nemoria originated in the Neotropics and subsequently radiated into North America, and they indicate that this may have occurred in at least seven independent colonization events. Current distributions can be explained by a series of range expansions and vicariant speciation events. Further work on historical biogeography and trait evolution in Nemoria will be crucial in understanding ecological interactions that may have shaped the evolution of phenotypic plasticity in this genus.

The estimate of the age of Nemoria presented in this study of approximately 7.5 ± 0.06 million years is tentative. We realize there are many shortcomings in using only uncorrected pairwise distances to resolve accurate ages. Without fossil or biogeographic information to help calibrate molecular data, dating divergence times for lineages can be challenging. We present the first estimate for the age of the genus, but wait for a more accurate date for the genus as more comprehensive future studies investigate this diverse group. This tentative date for the origin of Nemoria falls well after the recent estimate of the divergence time of geometrid moths (54 Mya), the Geometrinae (42 Mya) and also the range of divergences of winter moths (34–12 Mya) given by Yamamoto and Sota (2007).

Our results begin to shed light on the biogeography of at least one Nemoria species. Nemoria outina is endemic to the sand pine scrub habitat of central Florida and southern Georgia, and is a specialist on C. ericoides (Empetraceae) that also is endemic to that region. Since the sand pine scrub habitat did not appear before approximately 20 Mya (Webb, 1990), N. outina seems to have evolved well after the appearance of this habitat. Although more thorough historical biogeography analysis and dating for this species was beyond the scope of this research, these data suggest that additional population level information might provide interesting insights into the evolution of this species.

4.3. The evolution of phenotypic plasticity in Nemoria

Examples of phenotypic plasticity in both larval and adult forms are known in many of the major groups of Lepidoptera, but only a few of these have been examined in a phylogenetic context. In Araschnia and Bicyclus, there is evidence that phenotypic plasticity and seasonal forms are evolutionarily labile (Fric et al., 2004; Roskam and Brakefield, 1996).

Species that exhibit adult phenotypic plasticity in Nemoria are contained in at least three different major clades on the phylogeny. N. bistriaria, N. bifilata and N. lixaria are nested in a clade with other species that do not show seasonal variation, and they also represent two different kinds of variation, with N. lixaria displaying melanic variation and the other two having distinct green and brown forms (Fig. 3). Nemoria elfa, which has green and brown forms, is contained in a clade with three other southeastern species, none of which exhibits adult seasonal variation. In addition, N. arizonaria and N. viridicaria are recovered as sister taxa in this study. Plasticity in N. arizonaria appears to be different in composition from the green and brown forms of N. viridicaria, and it is possible that these are the result of a single origin of seasonal plasticity that later differentiated, or that it is actually the result of two independent evolutionary events due to similar selection on species experiencing similar ecological conditions. Unfortunately, we were unable to obtain material for N. pulcherrima and N. daedalea, which both exhibit adult plasticity, and therefore have no information about their phylogenetic placement (although Ferguson’s (1985) taxonomic review places N. daedalea in his species group II with N. arizonaria and N. viridicaria, and N. pulcherrima in his species group I with N. unitaria). Ancestral character reconstruction results obtained from Mesquite suggest four to six separate evolutions of adult phenotypic plasticity.

Phenotypic plasticity in larvae has been demonstrated in both N. arizonaria (McFarland, 1988; Greene, 1989, 1996) and N. outina (Canfield, 2006, see Fig. 3). In N. arizonaria, the differences in larvae are cued by seasonal differences in host plant, whereas in N. outina the larval forms are determined by the age of the host plant leaves, and light also has an effect on these forms (Canfield, 2006). These two species each have two larval forms that are extremely different in pigmentation and morphology, and that match two different cryptic microhabitats on their host plants. In the phylogenetic analysis of Nemoria, these two species are contained in different clades. The closest relatives of N. outina are a group of southeastern species, whereas those of N. arizonaria are southwestern and Neotropical Nemoria. Other species such as N. darwiniata (Greene, unpublished data), N. bifilata, N. cachaloha and N. bistriaria also exhibit larval variation and suggest that this characteristic is not limited to one or two parts of the Nemoria phylogeny.

The phylogenetic hypothesis presented here suggests multiple evolutionary origins of larval plasticity in Nemoria. The ancestral character reconstruction results obtained from Mesquite suggest phenotypic plasticity evolved at least six times in larvae. This in turn suggests that a similar underlying set of developmental and morphological factors may have allowed species in a number of clades to adapt to different ecological conditions. However, the larvae of many species of Nemoria are completely unknown or have received only a cursory treatment, and it is possible that phenotypic plasticity is much more common, such that the multiple instances of gain in plasticity observed here may just be the result of incomplete sampling from a larger clade of moths with variable larvae. Life history information for species whose biology is as yet unknown, combined with additional analyses of this and other groups will provide greater insight into factors influencing the evolution and maintenance of phenotypic plasticity.

Acknowledgments

The authors thank the Instituto Nacional de Biodiversidad (INBio) in Costa Rica and the University of Maryland for the generous use of specimens for this study. Special thanks to John Gruber and numerous members of the Lepidopterists’ Society including Brian Scholten, Dave Wagner, John Brown and Jerry Powell. We also thank Jesús Ugalde, Isidro Chacon and Richard Sobonya. Swee Peck Quek assisted with data analysis, and Dan Janzen and Dave Wagner provided logistical support. This research was supported by an NSF Graduate Research Fellowship and a Putnam Expeditionary Grant (Museum of Comparative Zoology) to M.R.C., and NSF DEB-0447242 to N.E.P.

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