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

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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, Geometroidea: 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*, *Lissochlora* 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.

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

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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 genitalic 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 from their host plant, *Ceratiola ericoides* (Empetraceae) (Canfield, 2006). Several other species such as *N. darwiniata* (Greene, unpublished data), *N. bifilata*, *N. catachloa* and *N. bistriaria* (Canfield, 2006) 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. elfa*, 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: Nemoriini, Synchlorini, Lophochoristini and Hemitheini.

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 $MgCl_2$, 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

Species	Species groups	Geographic range	Collection #	*COI* Acc. #	*COII* Acc. #	*EF-1α* Acc. #
N. unitaria	*I*	NW CAN S through CA, AZ, NM, SD, UT	JG05–C700	EU151594	EU151657	
N. albaria	*II*	AZ	EG03–B851	EU151577	EU151640	EU151680
N. arizonaria	*II*	AZ, TX, NM	EG03–B815	EU151575	EU151638	EU151678
N. viridicaria	*II*	CO, NM, UT, TX	EG03–B856	EU151576	EU151639	EU151679
N. diamesa	*II*	CO, NM, AZ	EG03–B817	EU151569	EU151632	EU151672
N. pistacearia	*III*	OR S through CA	MRC01–C187	EU151570	EU151633	EU151673
N. outina	*IV*	FL	MRC02–C209	EU151574	EU151637	EU151677
N. catachloa	*IV*	FL, GA	MRC02–C204	EU151573	EU151636	EU151676
N. elfa	*IV*	FL, NC, TX, KY, SC	MRC01–C100	EU151571	EU151634	EU151674
N. tuscarora	*IV*	NC, V, WV, KY	MRC02–C299	EU151572	EU151635	EU151675
N. saturiba	*V*	FL, NC, VA, KY, AL, MS, TN, TX	MRC01–C189	EU151582	EU151645	EU151685
N. lixaria	*V*	FL W to TX and N to MD	MRC01–C190	EU151581	EU151644	EU151684
N. darwiniata	*VI*	NW CAN S to S CA, W to Rocky Mts.	EG00–G005	EU151580	EU151643	EU151683
N. obliqua	*VI*	OR S through CA to N MEX, TX, NM, AZ, CO UT, NV	EG00–G002	EU151567	EU151630	EU151671
N. oblique hennei	*VI*	NM, AZ, CO, UT, NV, CA, OR	JG05–C701	EU151568	EU151631	
N. leptalea	*VIII*	N CA S through N MEX	MDLEP1139	EU151557	EU151620	EU151661
N. glaucomarginaria	*VIII*	CA, OR, WA	JT04–C308	EU151558	EU151621	EU151662
N. bifilata	*VIII*	NJ S through FL, W to LA, TX	MRC03–C203	EU151579	EU151642	EU151682
N. mimosaria	*VIII*	Nova Scotia W to Manitoba and SD, S to LA, MS, TX	MRC01–C149	EU151563	EU151626	EU151667
N. rubrifrontaria	*VIII*	Nova Scotia W to Ontario, S to NC, W to KA, KY, MO	MRC05–C303	EU151559	EU151622	EU151663
N. bistriaria	*VIII*	QUE and ONT S to TX, MO	MRC01–C161	EU151562	EU151625	EU151666
N. festaria	*VIII*	TX, AZ	EG03–B829	EU151561	EU151624	EU151665
N. caerulescens	*VIII*	TX, NM, AZ, CO	EG03–B845	EU151560	EU151623	EU151664
N. karlae	*cosmeta*	Neotropical MEX to Panama	MRC03–K183	EU151556	EU151619	EU151660
N. lorenae	*cosmeta*	Guatemala and NW Costa Rica	CRI0002195401	EU151607		
N. punctilinea	*erina*	Neotropical MEX; Costa Rica to Brazil, Bolivia and French Guiana	MRC03–K127	EU151588	EU151651	EU151691
N. erina	*erina*	Mexico to Brazil, French Guiana and Bolivia	INB0003401536	EU151612		
N. tutala	*pacificaria*	MEX to French Guiana, Ecuador and Brazil	CRI000939943	EU151599		
N. pacificaria	*pacificaria*	Neotropical MEX to French Guiana	CRI000928189	EU151615		
N. adjunctaria	*pulveraria*	Guatemala to Colombia	MRC03–K210	EU151590	EU151653	EU151693
N. tickelli	*pulveraria*	Costa Rica	MRC03–K151	EU151591	EU151654	EU151694
N. anae	*pulveraria*	Two locales in Costa Rica	INB0003088162	EU151601		
N. epaphras	*pulveraria*	Costa Rica and Panama	INB0003116365	EU151598		
N. eugeniae	*pulveraria*	Costa Rica	INB0003487344	EU151595		
N. scriptaria	*scriptaria*	Costa Rica to Brazil, Bolivia and French Guiana	MRC03–K201	EU151589	EU151652	EU151692
N. elbae	*scriptaria*	Costa Rica	INB0003154025	EU151610		
N. ozalea	*scriptaria*	Costa Rica	INB0003496201	EU151611		
N. strigaria	*strigaria*	Costa Rica	CRI002550531	EU151614		
N. saryae	*strigaria*	Costa Rica	CRI002550439	EU151613		
N. dentilinea	—	Costa Rica to French Guiana, Brazil and Bolivia	MRC03–K206	EU151564	EU151627	EU151668
N. nympharia	—	Guatemala and Costa Rica	RE01–H374	EU151566	EU151629	EU151670
N. acutularia	—	Costa Rica	MRC03–K78	EU151554	EU151617	EU151658
N. callirrhoe	—	Costa Rica to French Guiana and Colombia	MRC03–K211	EU151578	EU151641	EU151681
N. defectiva	—	Costa Rica, Colombia and Peru	MRC03–K102	EU151565	EU151628	EU151669
N. vermiculata	—	Costa Rica and Colombia	INB0003325572	EU151596		
N. astraea	—	MEX to Venezuela and Peru	INB0003703001	EU151603		
N. carolinae	—	Guatemala to Trinidad, Peru and Brazil	INB0003078475	EU151604		
N. dorsilinea	—	Costa Rica	INB0003334453	EU151600		
N. venezuelae	—	Guatemala to Brazil and Peru	INB0003080856	EU151597		
N. winniae	—	Costa Rica	INB0003520603	EU151616		
N. remota	—	MEX to Colombia	INB0003445743	EU151606		
N. rectilinea	—	MEX to Panama, Cuba and Dominica	CRI002573377	EU151608		
N. vinocincta	—	Costa Rica and Panama	CRI002014910	EU151602		
N. pescadora	—	MEX and Costa Rica	CRI002358111	EU151609		
N. florae	—	Costa Rica and Ecuador	CRI000809258	EU151605		
Chlorochlamys chloroleucaria 1	—	Nova Scotia to Manitoba, S through USA to Bahamas, Cuba and MEX	MRC02–C205	EU151585	EU151648	EU151688
Hethemia pistasciaria	—	Nova Scotia to Manitoba, ND, MO S to FL, AL	MRC01–C150	EU151587	EU151650	EU151690
Lomographa semiclarata	—	CAN S to MO and SD	MRC02–C238	EU151593	EU151656	EU151696
Dyspteris arbortivaria	—	Throughout USA	MRC02–C285	EU151592	EU151655	EU151695
Synchlora frondaria	—	Southeastern USA to Central and South America	MRC02–C279	EU151584	EU151647	EU151687
Chlorochlamys chloroleucaria 2	—	Nova Scotia to Manitoba, S through USA to Bahamas, Cuba and MEX	MRC02–C301	EU151586	EU151649	EU151689
Oospila venezuelata	—	MEX S to Colombia	MRC03–K202	EU151555	EU151618	EU151659
Synchlora aerta	—	Throughout USA and CAN	EG00–G007	EU151583	EU151646	EU151686

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.

reactions kits (Applied Biosystems, CA). Primers for sequencing were the same set as used for amplification, although internal primers were sometimes used for longer fragments. All fragments were sequenced in both the forward and reverse direction

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

Sequences were aligned using Sequencher 3.1.1 (Genecodes, Ann Arbor, MI) in comparison with total mtDNA sequence (GenBank No. NC_002355, Lee and Kim, 1999) and *EF-1α* 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-1α* (39 taxa) were analyzed separately and then all data from *COI*, *COII* and *EF-1α* (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 Fujiwara, 1993; Kuhner and Felsenstein, 1994; Huelsenbeck, 1995).

Parsimony heuristic searches in PAUP* 4.03b10 (Swofford, 2001) using equal character weighting consisted of 1000 replicates with random stepwise addition, tree 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-1α* 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* 4.03b10. 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-clocklike) ($P < 0.001$), 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; Schubart 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

Table 2
Primers used in gene amplification

Primer	Sequence	*D. yakuba* Ref.	Primer citation
COI and COII			
LCO-1490	5′-GGTCAACAAATCATAAAGATATATTGG-3′	1490	Folmer et al. (1994)
HCO-2198-r	5′-TAAACTTCAGGGTGACCAAAAAAATCA-3′	2198	Folmer et al. (1994)
Ron	5′-GGATCACCTGATATAGCATTCCC-3′	1730	Simon et al. (1994), Monteiro and Pierce (2001)
Nancy-r	5′-CCCGGTAAAATTAAAATATAAACTTC-3′	2217	Simon et al. (1994), Monteiro and Pierce (2001)
Tonya	5′-GAAGTTTATATTTTAATTTTACCGGG-3′	2192	Monteiro and Pierce (2001)
Hobbes-r	5′-AAATGTTGNGGRAAAAATGTTA-3′	2757	Monteiro and Pierce (2001)
JL2532	5′-ACAGTAGGAGGATTAACAGGAG-3′	2532	R. Eastwood (personal communication)
JL3146	5′-GAGTTTCACCTTTAATAGAACA-3′	3146	R. Eastwood (personal communication)
TN2126	5′-TTGAYCCTGCAGGTGGWGGAGA-3′	21266	R. Eastwood (personal communication)
George	5′-ATACCTCGACGTTATTCAGA-3′	2774	Monteiro and Pierce (2001)
Phyllis-r	5′-GTAATAGCIGGTAARATAGTTCA-3′	3298	Monteiro and Pierce (2001)
Strom	5′-TAATTTGAACTATYTTACCIGC-3′	3271	Monteiro and Pierce (2001)
Eva-r	5′-GAGACCATTACTTGCTTTCAGTCATCT-3′	3799	Monteiro and Pierce (2001)
BtLYS-r	5′-GTTTAAGAGACCAGTACTTG-3′	3808	Simon et al. (1994), Monteiro and Pierce (2001)
EF-1α		*Bombyx mori*	
ef44	5′-GCYGARCGYGARCGTGGTATYAC-3′	240	Monteiro and Pierce (2001)
ef51.1-r	5′-CATGTTGTCGCCGTGCCAAC-3′	650	Monteiro and Pierce (2001)
ef51.9	5′-CARGACGTATACAAAATCGG-3′	798	Monteiro and Pierce (2001)
efrcM4	5′-ACAGCVACKGTYTGYCTCATRTC-3′	1351	Monteiro and Pierce (2001)
ef46.1	5′-CGAGGAAATCAARAARGAAG-3′	548–567	Cho et al. (1995)
ef52.6-r	5′-GCTTCGTGGTGCATTTCAAC-3′	940–921	Cho et al. (1995)

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 make 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 (λ_D) and the rate of extinction within an area (λ_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-1*α) 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-1*α 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: A⇔C 7.75717; A⇔G 22.55089; A⇔T 3.02547; C⇔G 5.46392; C⇔T 92.73226; G⇔T 1.0 and base composition was A = 0.39239; C = 0.09238; G = 0.08654; T = 0.42868, with $\alpha = 0.387$. The *EF-1*α tree had a score of $-\ln L = 5054.85199$ and the GTR relative rate parameters were: A⇔C 1.98196; A⇔G 7.00989; A⇔T 4.11520; C⇔G 1.01496; C⇔T 20.05116; G⇔T 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: A⇔C 4.93891; A⇔G 24.80498; A⇔T 10.89298; C⇔G 3.69035; C⇔T 51.72075; G⇔T 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: A⇔C 5.37439; A⇔G 26.87757; A⇔T 11.55195; C⇔G 3.78612; C⇔T 59.89581; G⇔T 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.,

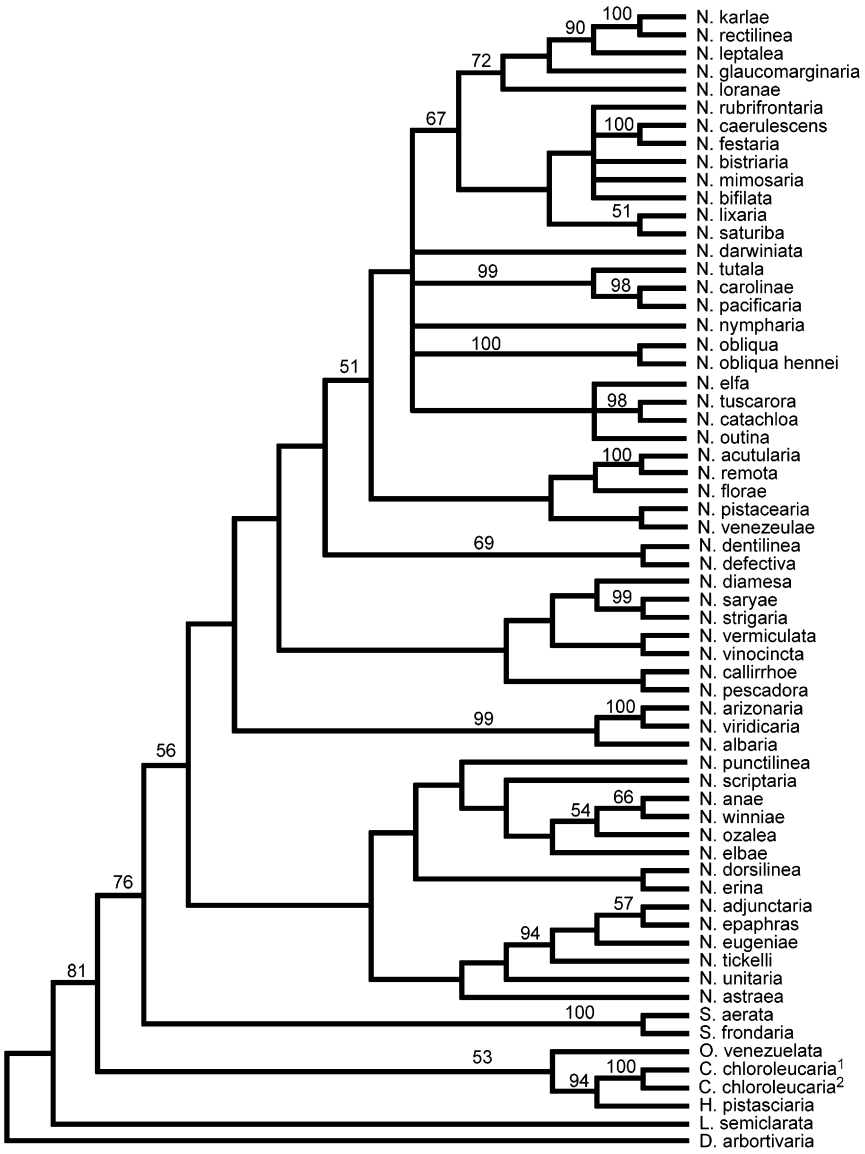

Fig. 1. Strict consensus tree from five most parsimonious trees using the combined *COI* + *COII* and *EF-1α* data set. Numbers above nodes represent bootstrap values above 50%.

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. catachloa* + *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. remota* + *N. dentilinea* + *N. defectiva* + *N. florae*, bpp = 95%; *N. tutala* + *N. pacificaria* + *N. carolinae*, 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. rectilinea* 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. rubrifrontaria*, *N. bistriaria*, *N. mimosaria*, *N. saturiba*, *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*.

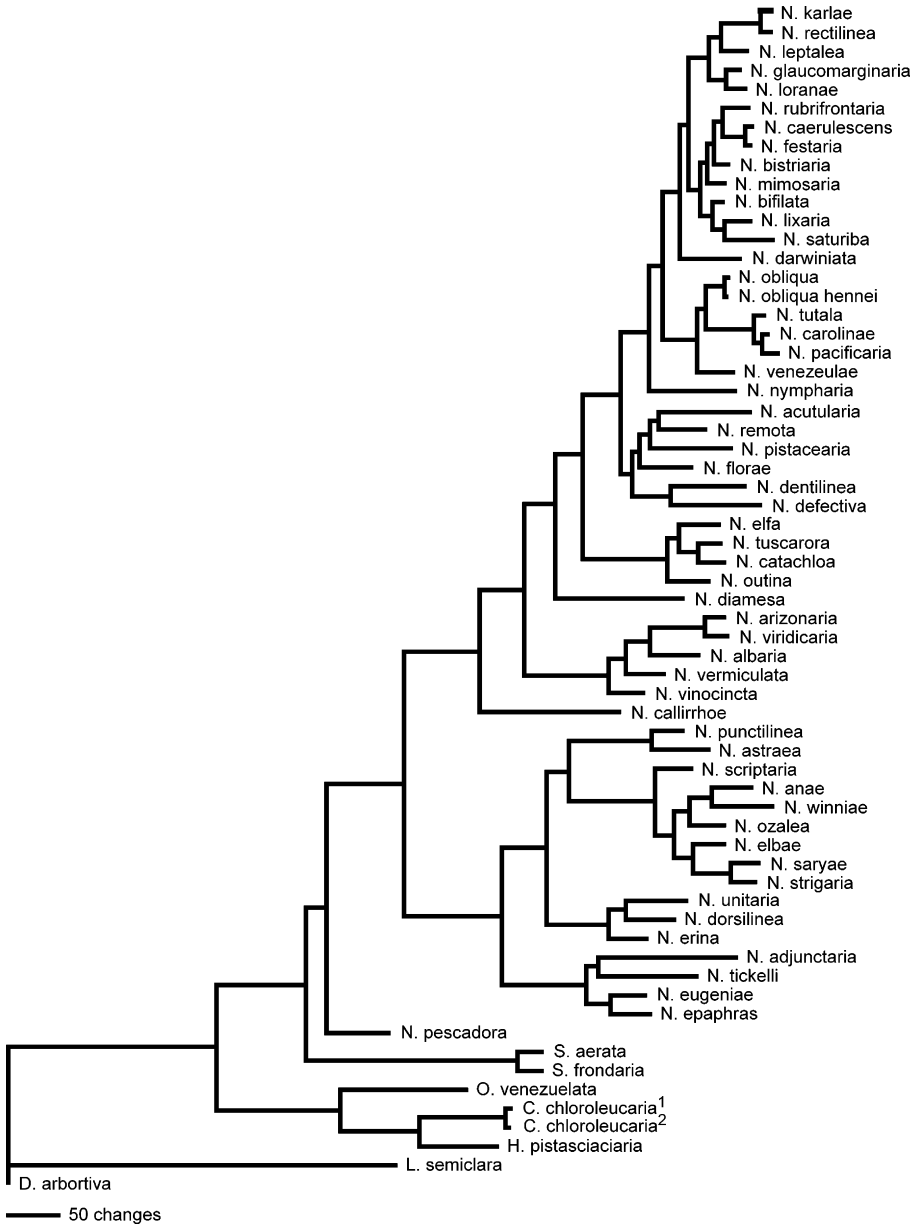

Fig. 2. Maximum likelihood tree with proportional branch lengths. Based on a GTR+Γ model of sequence evolution.

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 genitalic 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-

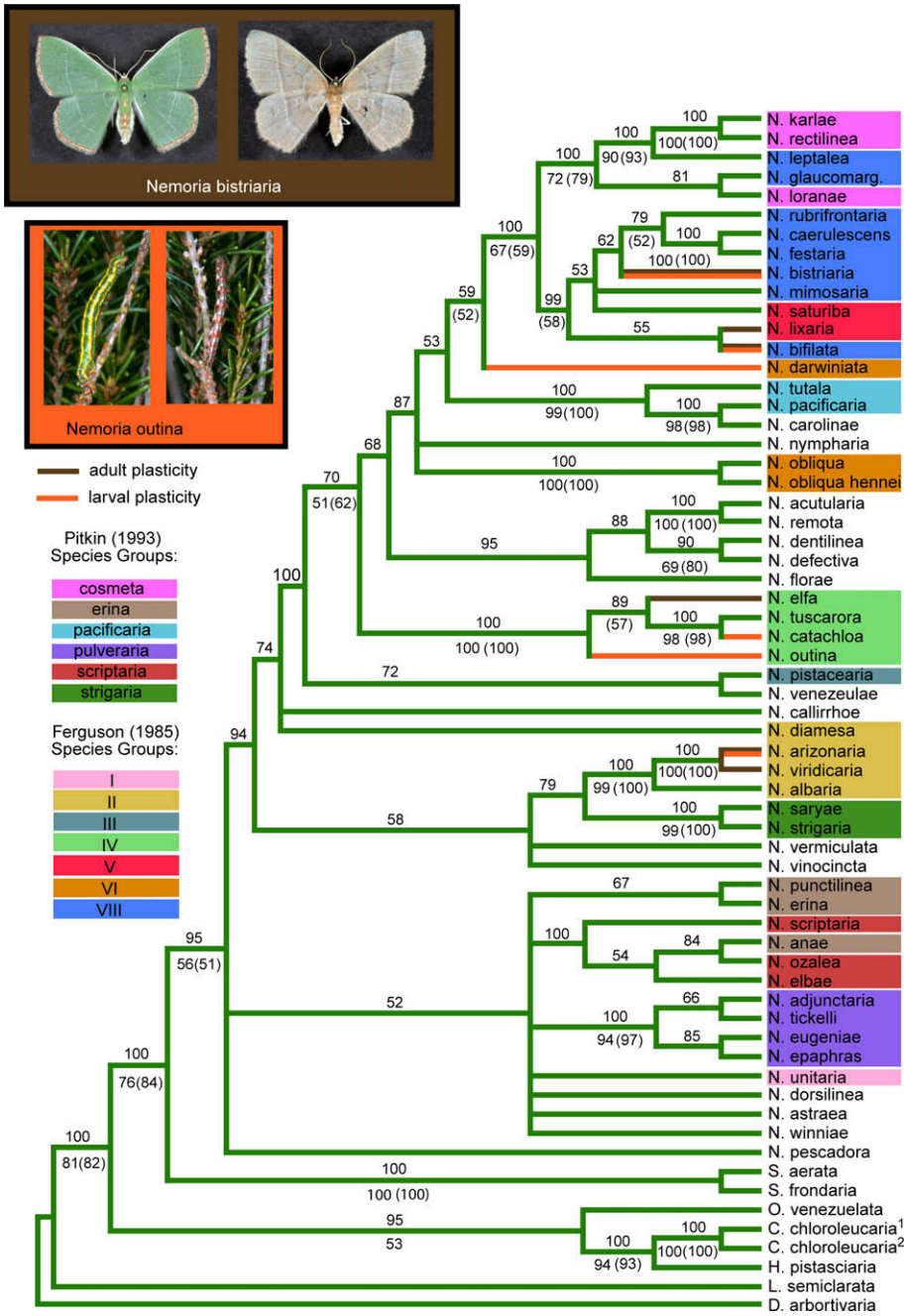

Fig. 3. Bayesian inference tree (10,000,000 generations) of the combined *COI + COII + EF-1α* data set using a GTR+Γ model of sequence evolution. Posterior probabilities are shown above nodes. Bootstrap values of nodes supported in both the maximum parsimony (below nodes) and maximum likelihood analysis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

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 *pacificaria*, *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. pacificaria* (bpp = 100%, MP bs = 99%, ML bs = 100%) indicates that *N. carolinae* belongs in the *pacificaria* 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.

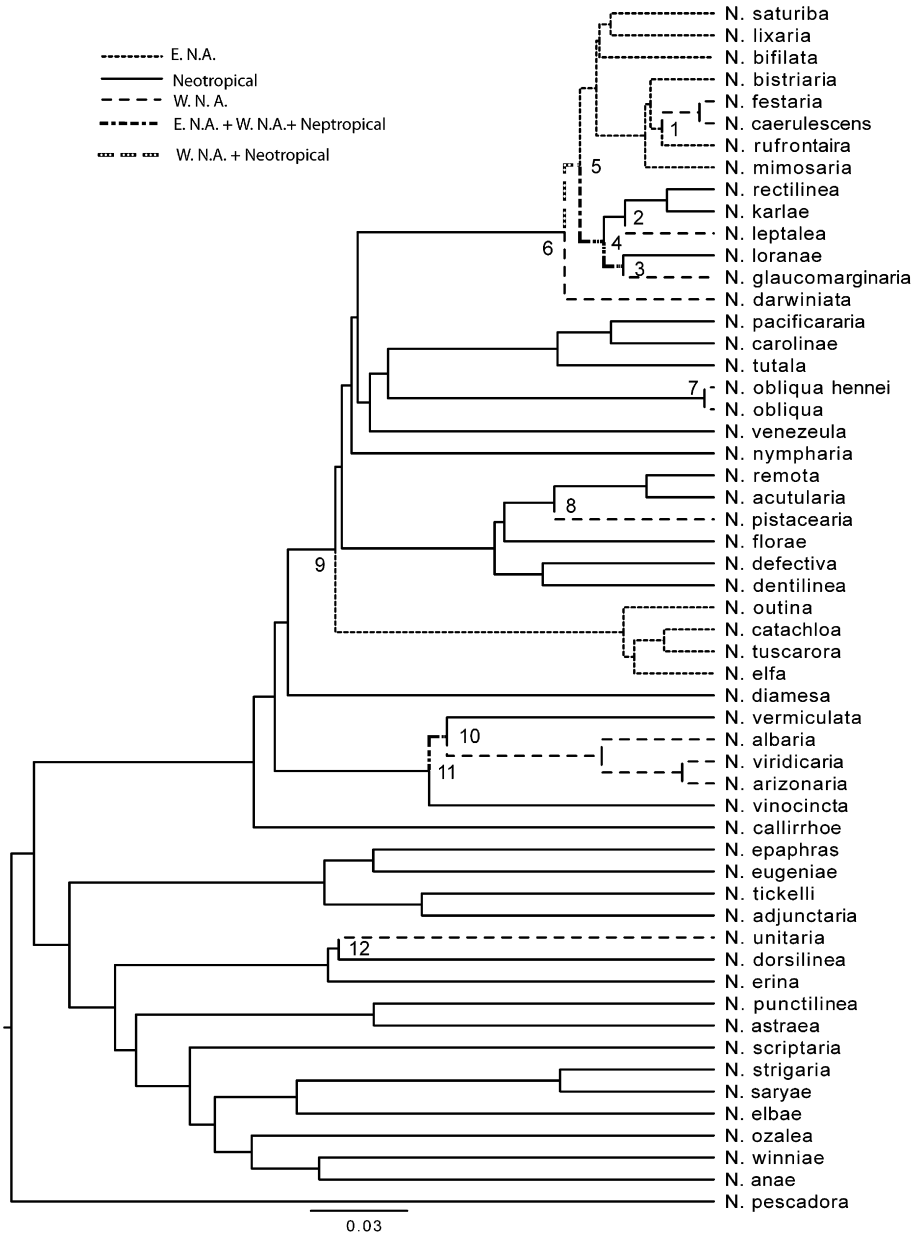

Fig. 4. LAGRANGE results using a likelihood framework. Numbers at nodes correspond to independent colonization events. Results show the highest likelihood geographic distributions of each node and taxon.

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. karlae*, *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. tuscarora*, *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. mimosaria*. 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 *Arashnia* 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. catachloa* 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.

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