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Phylogeographic analysis of a recent radiation of *Enallagma* damselflies (Odonata: Coenagrionidae)

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Abstract

A phylogenetic hypothesis revealed two recent radiations among species of *Enallagma* damselflies, and extensive ecological work suggests that both adaptive and nonadaptive processes are involved in these radiations. We analysed the geographical pattern of genetic variability at 868 bp of mitochondrial DNA (mtDNA) among 283 individuals of 5 species displaying little ecological differentiation to identify the ancestral lineage, support their independent evolutionary trajectories and identify historical events and the underlying mechanism for one of these radiations. Nested clade analysis results clearly support a past event of range fragmentation in *E. hageni*. These Atlantic and Continental *hageni* races experienced distinct dispersal histories and still maintain nearly nonoverlapping ranges All four other species derive from the Continental *hageni*. Whereas three species endemic to the Atlantic coastal plain show little genetic variation, *E. ebrium* shared several haplotypes with the Continental *hageni*. Contrasting levels of genetic differentiation between *E. hageni* and *E. ebrium* in geographical areas associated with distinct events of *E. hageni*'s recent history support the recent origin of this species. Altogether, our results are compatible with a process of radiation via divergence in mate recognition systems within the Continental *hageni* race following secondary contacts between putative refugial races.

Keywords: *Enallagma*, mtDNA, nested clade analysis, phylogeography, radiation

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Introduction

The process of biological radiation involves the rapid diversification of a single lineage into multiple species, or at least cohesive genetic groups, which then each assume independent evolutionary trajectories. The nature of the traits discriminating species in a radiating clade is often used to infer the mechanism or mechanisms that have promoted or permitted diversification to occur. On these bases, radiations are generally classified as adaptive or nonadaptive. In adaptive radiation, the array of species differs in phenotypic traits that confer advantages for the exploitation of different features of the ecological environment (e.g. Schluter 2000), and ecological diversification is readily apparent.

In contrast, nonadaptive radiation gives rise to species that are primarily differentiated by characters that do not improve their ability to utilize any particular segment of an adaptive zone (e.g. ploidy levels, chromosomal arrangements, secondary sexual characters), and ecological diversification is not apparent. Although this nonadaptive view of radiation has traditionally received less attention, evidence from recent studies suggest that divergence in reproductive phenotypes has indeed played a critical role in several radiations (Kaneshiro 1983; Henry 1985; Shaw 1996a,b; Seehausen *et al*. 1997, 1999). Whereas most authors stress the prevalence of one type of radiation mechanism, adaptive or nonadaptive, these processes are not inherently mutually exclusive in any given radiation. For example, sequential radiations involving an ancient adaptive divergence in cichlid jaw morphology followed by a recent bout of sexual radiation based of female colour preference is now becoming the accepted view of the processes that drove the African mbuna cichlids radiations (Albertson *et al*. 1999; Danley & Kocher 2001).

Identifying and discriminating the driving forces triggering bursts of divergence goes beyond the mere theoretical interest for evolutionary biologists, as it may be pertinent to gauge the relevance of the biodiversity resulting from these different processes. Adaptive radiations are inherently ecologically dependent; their potential for

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diversification is thus limited by a conceivably finite number of potential niches, but each resulting species bears a unique ecological relevance. The potential for diversification is higher in nonadaptive radiation, as the number of resulting species is not limited by the number of available ecological opportunities and myriad character values for diverging traits can give reproductive isolation, especially in sexual radiation. However, the resulting species may be ecologically highly redundant (Walker 1992; Tilman & Pacala 1993; McCann 2000), and analysis of such 'hot spots' of biodiversity may call for neutral models to understand patterns of biodiversity (e.g. Hubbell 2001).

Enallagma damselflies are among the most diverse genera of aquatic insects in North America (Westfall & May 1996). A phylogenetic hypothesis for *Enallagma* species of North America indicates that the genus is composed of two primary clades with very different evolutionary histories (Brown *et al*. 2000; McPeek & Brown 2000). In one clade, regional species richness is greatest in the southeastern USA, and both recent and relatively old divergences are observed among species. In the other clade, species richness reaches its maximum in northeastern USA, and the very close genetic relationships among several species reveals two parallel and very recent radiations whereby two lineages have each given rise to 7 and 10 species, respectively (Brown *et al*. 2000; McPeek & Brown 2000).

Various lines of evidence suggest that both adaptive and nonadaptive processes have influenced speciation events within each of these two recently radiated clades. Ecological diversification in the form of a major habitat shift played a substantial role in both of these radiations. Most species within each radiation are found as larvae only coexisting with fish predators, but two species in each radiation are found as larvae only in fishless ponds and lakes, in which large dragonflies are the top predators (McPeek 1990a, 1998; Brown *et al*. 2000; McPeek & Brown 2000). Phylogenetic reconstruction of these habitat affinities indicates at least one habitat shift from the ancestral fish-lake habitat within each radiation, and character reconstructions also clearly show that major evolutionary changes in morphology, physiology and behaviour were associated with adapting to living with dragonfly predators (McPeek 1990b, 1995, 1996, 1997, 1999, 2000; McPeek *et al*. 1996).

In contrast to these adaptive habitat shifts, most species in both radiations were produced by processes operating within the ancestral fish-lake habitat. Ecological diversification does not seem to have played any significant role. Up to 12 species can be found co-occurring at any given fish lake in eastern North America, and no microhabitat differentiation of *Enallagma* species within fish lakes is apparent (Johnson & Crowley 1980; McPeek 1990a,b, 1998). Moreover, these co-occurring fish-lake species are all phenotypically very similar, they all experience similar mortality rates from fish predators, and they grow at similar rates in the field (McPeek 1990a,b, 1995, 1998, 1999, 2000; McPeek *et al*. 1996, 2001). As adults, many of these species also have coincident or overlapping flight and mating seasons (Westfall & May 1996; McPeek & Turgeon, personal observation).

This study is part of a global investigation on the relative contribution of adaptive and nonadaptive processes that have affected and still govern patterns of regional species richness and local species assemblages of *Enallagma*. Noting that the highest species richness in both radiations lies in a region that was only recently deglaciated (New England), we hypothesize that historical factors linked to the recolonization process may have played a role in these radiations. To exclude the confounding effect of adaptive processes (see above), we focus on five species from one of the radiations that all inhabit lakes with fish and that show little, if any, ecological differentiation. We examine the phylogeographical pattern of genetic variation within and among these species the answer the following questions:

1 Which, if any, of these species represents the ancestral lineage(s) for this radiation?
2 Can geographical information be coupled to patterns of genetic relatedness to identify historical events supporting the recent independent evolutionary trajectories for the ancestral/derived species?
3 Can geographical information suggest the location and mechanism for this radiation?

Materials and methods

Biological material and sequencing procedures

Two hundred and eighty-three adult males of *Enallagma hageni*, *E. ebrium*, *E. recurvatum*, *E. minusculum* and *E. laterale* were collected from 58 sites between 1995 and 2001 (Table 1, Fig. 1), covering most of each species range in North America. *E. hageni* and *E. ebrium* are widely distributed species, ranging from the Atlantic coast of North America to Alberta and the very eastern edge of British Columbia (Westfall & May 1996). In contrast, *E. recurvatum*, *E. minusculum* and *E. laterale* are all endemic to the Atlantic coastal plain (Westfall & May 1996). All individuals were unequivocally identified to species by us on the basis of caudal appendage morphology using Westfall & May (1996). Upon collection, specimens were either dried over a desiccant or frozen in liquid nitrogen and then stored at −80 °C. DNA was extracted from the thorax with diatomaceous earth and guanidine thiocyanate, using the protocol of T. Glenn (Savannah River Ecology Laboratory, GA; http://www.uga.edu/srel/DNA_Laboratory/protocols.htm). The abdomens of all specimens were preserved in 70% EtOH as vouchers for the caudal appendage morphology.

Table 1 Desription and geographical coordinates of sampling sites for *Enallagma hageni* (H), *E. ebrium* (E), *E. laterale* (L), *E. minusculum* (M) and *E. recurvatum* (R) and haplotypes (see Fig. 2) documented in each site and species

				Species					
Code	Sampling location Waterbody, town	Lat.	Long.	H	E	L	M	R	Haplotypes (*N* if > 1)
BC1	Pelican Lake, Prince George	54.00	123.00		3				E: 5, 24(2)
BC8	MurdoughLake	52.75	119.20	1	5				H: 24 E: 24(3), 38, 39
AB4	Ketchmark # 1, Brooks	52.50	114.20		2				E: 21(2)
AB12	Chickakoo Lake, Stony Plain	52.30	114.00	5	5				H: 21(4), 24 E: 21(3), 36, 37
SK1	Trappers Riv., P. Edward Natl Park	53.85	106.10	4					H: 102, 103(2), 104
ID1	Brush Lake, Boundary Co.	48.89	116.33		4				E: 21(2), 40(2)
MN1	Low Lake, Ely	47.98	91.82	5					H: 2(4), 61(1)
WI2	Minnesuing Lake, Solon Springs	46.47	91.74	1					H: 101
WI3	Highway 2 pond, 2 km NW of Brule	46.57	91.63		1				E: 41
WI4	Gilbert Lake, Brule State Forest	46.42	91.80	2	2				H: 26(2) E: 41 (2)
MI1	Kennedy Lake, Germfask	46.21	85.89	7					H: 13, 31(3), 54, 61(2)
MI2	Little Brevoort Lake, Moran	46.02	85.02	2					H: 24, 60
MI3	Strouble Lake, Naubinway	46.14	85.19	4					H: 13, 57, 58(2)
MI4	Three Lakes II, Richland	42.35	85.43	8					H: 59(4), 60, 61(2), 62
MI5	Pond Lab, KBS, Hickory Corners	42.41	85.39		3				E: 41(3)
MI13	George's Reserve, Pinckney	42.45	84.00		2				E: 41(2)
PA4	Peck's Pond, Milford	41.28	75.09			7			L: 30(7)
PA5	State Game Area 219, Erie County	42.04	79.92		1				E: 35
NY1	Crystal Lake, Starbuckville	43.65	73.74	18	4				H: 35(15), 63(3) E: 35(4)
NY3	Moss Lake, Houghton	42.40	78.19		5				E: 35(4), 41
NY6	Mosher Pond, Pulaski	43.47	76.01		4				E: 35(3), 41
VT1	Amhearst Pond, Plymouth	43.49	72.71	3					H: 67, 69, 70
VT8	Bresee Pond, Hubbardton	43.71	73.22		4				E: 35(4)
VT9	L. Ninevah, Grahamville	43.47	72.75		2				E: 35(2)
VT11	Hough Pond, Sudbury	43.78	73.18		4				E: 35(4)
VT13	Elfin Pond, Wallingford	43.47	72.99		1				E: 35
VT16	Little Salem Pond, Newport	44.92	72.10	4					H: 41, 50, 53(2)
VT18	Spectacle Pond, Island Pond	44.80	71.86	4					H: 21, 51, 52, 53
VT19	Martin's Pond, Green Bay	44.30	72.21	17					H: 67(13), 35, 53(3)
VT20	Ticklenaked Lake, Boltonville	44.19	72.10	4					H: 67(2), 71(2)
NH1	Moose Mountain Pond, Etna	43.70	72.16		4				E: 34, 35 (2), 41
NH2	McDaniel's Marsh, Enfield	43.53	72.08		4				E: 41(4)
MA1	Herring Pond, Wellfleet	41.82	69.98			4			L: 29(4)
MA2	Horseleech Pond, Truro	41.97	70.00				4	4	M: 31(4) R: 31, 32(3)
MA5	Lovell's Pond, Marston	41.65	70.45			4			L: 27, 28(2), 29
MA8	Little Cliff Pond, Brewster	41.76	70.02					4	R: 31(3), 33
NJ1	Basto Pond, Peasant Mill	39.65	74.65					4	R: 31(4)
NJ3	Paper Mill Pond, Cape May Co.	39.24	74.90					3	R: 31(3)
NJ5	Lake Nummy, Cape May Co.	39.25	74.86			4			L: 30(4)
CT1	Wood Creek Pond, Norfolk	42.02	73.20			2			L: 29(2)
ME1	Lovewell Pond, Fryeburg	44.00	70.94	21					H: 64, 65, 67(15), 68(2), 72
ME2	Otter Pond, Bridgton	44.02	70.68			3			L: 30(3)
ME3	Perley Pond, Sebago	43.90	70.67			3	9		L: 29, 30(2) M: 31(9)
ME17	Drew Pond, Mee Corners	43.64	70.95		2				E: 35(2)
ME25	Dolby Pond, East Millisocket	45.67	68.63	3	3				H: 35(3) E: 41(3)
NB4	Lake George, Kings Landing	45.61	66.88				4		M: 2(4)
NB7	St. John River, Morrisdale	45.36	66.09	3					H: 53(3)
NS5	Nyanza, Cape Breton	46.10	60.93	3					H: 31(3)
NS9	Little Mushamush L., New Cornwall	44.50	64.49	4			4		H: 105, 106(2), 107 M: 2(2), 31(2)
PE1	Save Easy, Alberton	46.80	64.20	4	1				H: 2(2), 44, 45 E: 35
PE2	East Lake at Rte 16	46.40	62.00	4					H: 2(2), 46, 47
QC1	Lac Romulus, ZEC de la Blanche	47.40	72.10	5	4				H: 42(2), 48, 49, 53 E: 42(4)
QC2	DLL Pond, Pont-Rouge	46.75	71.67	1					H: 41
QC4	Lac à la Truite, Daveluyville	46.25	72.25	4					H: 35, 41(3)
ON2	Lake Katchewanooka, Peterborough	44.43	78.28		1				E: 35
ON3	Sawyer Creek, Lakefield	44.44	78.21			3			E: 35, 41(2)
ON6	Crane Creek Bog, Buckhorn	44.64	78.28	2					H: 35(2)
ON10	Bab Lake, Algonquin Provincial Park	45.63	78.42	3					H: 35(2), 43
	Total (N indiv.)			146	74	27	21	15	
	Total (N pop.)			28	25	7	4	4	

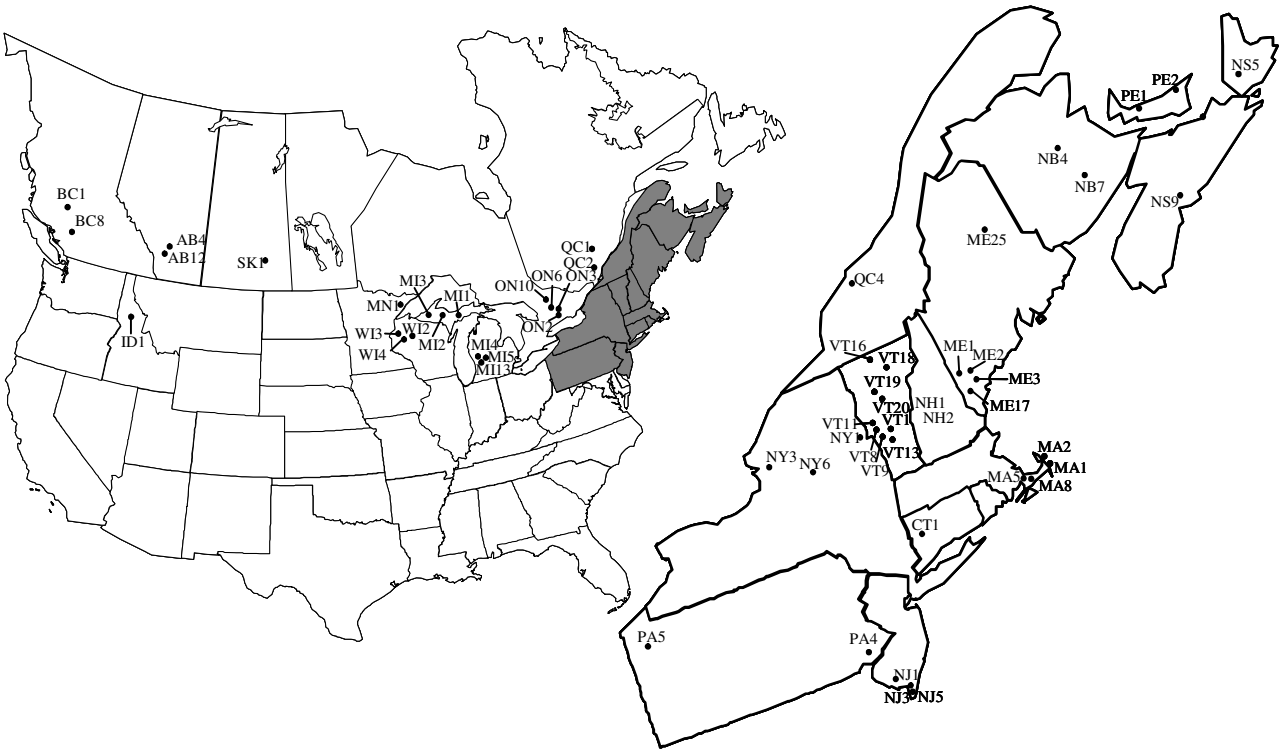

Fig. 1 Location of sampling sites for *Enallagma* species. Codes are as per Table 1.

For each individual, we obtained 884 bp sequences as described below. We first amplified an ≈ 1 kb segment of the mitochondrial genome spanning parts of COI, COII and the intervening leucine tRNA, as per Brown *et al*. (2000), but we used slightly modified primers sequences (COIs: 5′-CAGGAATACCTCGACGTTATTC-3′ and COIIa: 5′-CCAGTACTTGCTTTCAGTCATCT-3′). Polymerase chain reaction (PCR) products were purified either by precipitation with polyethylene glycol (T. Glenn, Savannah River Ecology Laboratory, GA; http://www.uga.edu/srel/DNA_Laboratory/protocols.htm) or by agarose gel extraction with the Quiagen Quick Gel Extraction Kit and protocol. These were sequenced directly using Applied Biosystems BigDye terminator chemistry. Sequences were assembled and aligned using Sequencher and GCG Wisconsin Package, respectively, providing unambiguous sequences of 868 bp for all 283 individuals.

Data analysis

We first attempted to establish the relationship among all documented haplotypes by performing a maximum parsimony analysis. The analysis was conducted using the heuristic search procedure of PAUP* Version 4.0b10 for Windows (Swofford 2002), using sequences of two species belonging to a sister clade, i.e. *E. durum* and *E. geminatum* (Brown *et al*. 2000) as outgroups. We assessed the support for resolved internal nodes by performing 100 bootstrapped pseudoreplicates.

We also established the mutational network relating all documented sequences and estimated the limit of validity of parsimonious relationships (Templeton *et al*. 1992; Templeton 1998) using TCS (Clement *et al*. 2000). This network was rooted using the same sequences as above. The three endemic species, *E. laterale*, *E. minusculum* and *E. recurvatum*, showed very little genetic variation and were not subjected to further analysis (see Results). By contrast, both *E. hageni* and *E. ebrium* globally displayed higher genetic diversity, and were intertwined in the global network (see Results). We used this global network to analyse and compare the geographical pattern of genetic variation in *E. hageni* and *E. ebrium*. For each species, haplotypes were nested into clades according to the rules presented in Templeton & Sing (1993), and nested clade analyses (NCA) were performed using GEODIS software (Posada *et al*. 2000). For higher nesting levels, tip vs. interior status was inferred using the outgroup method.

Results

Fifty variables nucleotide positions defined 57 distinct haplotypes among the 283 individuals sequenced. Figure 2 specifies the mutations found in each haplotype relative to a reference *Enallagma hageni* sequence (H2) that has been

							CO I																											CO II																							Species				
Seq #	1	26	45	71	73	84	90	122	125	137	146	152	167	273	285	308	312	336	342	343	357	363	367	417	461	474	480	489	496	507	513	522	555	591	606	615	621	627	643	651	660	681	714	720	723	732	759	781	804	816	817	822	828	834	852	H	E	L	M	R
H 2	C	A	C	T	C	G	G	G	T	T	G	T	A	T	A	T	T	C	C	A	A	A	G	C	T	T	A	A	T	A	G	A	G	T	A	G	A	C	T	G	T	A	C	T	A	A	G	A	G	A	C	T	A	C	T	*			*	
H 5	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.		*			
H 13	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*				
H 21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	*			
H 24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	*	*			
H 26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*				
H 27	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			*		
H 28	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.			*		
H 29	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			*		
H 30	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			*		
H 31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	*			*	*
H 32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.					*
H 33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.					*
H 34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	A	C	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.		*			
H 35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	C	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	*	*			
H 36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		*			
H 37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.		*			
H 38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.		*			
H 39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.		*			
H 40	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.		*			
H 41	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	*	*			
H 42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	*	*			
H 43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	C	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	*				
H 44	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	*				
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H 65	.	.	.	C	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	G	.	G	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	*				
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H 69	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	G	.	G	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	G	.	.	.	.	.	T	.	*				
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H 102	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	*				
H 103	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	*				
H 104	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	*				
H 105	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	*				
H 106	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	*				
H 107	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	*				

Fig. 2 Variable nucleotides for a 868 bp mitochondrial DNA fragment spanning parts of the cytochrome oxidase subunits I and II and the intervening leucine tRNA. The reference sequence (H2, site PE1) has been deposited in GenBank (Accession no. AF512684).

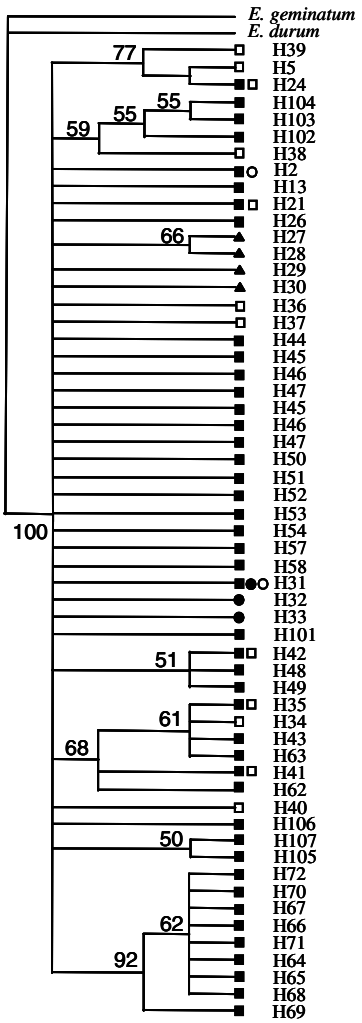

Fig. 3 Maximum parsimony majority-rule consensus tree at 50% bootstrap level among 868 bp mtDNA COI-II *Enallagma* sequences. Haplotype codes are those of Fig. 2 and symbols indicate in which species these haplotypes were found (see Fig. 4).

deposited in GenBank (Accession no. AF512684), whereas Table 1 indicates which haplotypes were found in each species and at each geographical location.

The strict consensus tree of the 48 most parsimonious trees was poorly resolved and contained numerous polytomies. The majority-rule consensus tree for 50% bootstrap values (Fig. 3) shows that none of the species formed a distinct clade. Only one clade was well-supported (BS > 90), and it contained a group of haplotypes found only in *E. hageni* (H64–H72). Whereas nearly half of the haplotypes defined a starburst tree structure, six small subclades were supported with bootstrap values above 50%. All contained haplotypes found in more than one species, except that grouping two haplotypes unique to *E. laterale*.

The mutational network relating the sequences found in all five species is presented in Fig. 4. There was little genetic variation among the three species restricted to the Atlantic coastal region, each being represented by at most four haplotypes that occupied restricted areas of the global network. *E. laterale* comprised four unique haplotypes that were not found in any other species (haplotypes H27–H30). *E. minusculum* and *E. recurvatum* were mainly characterized by a single common haplotype (H31) also documented in *E. hageni*. Two unique one-step derivatives of this haplotype were also found in some populations of *E. recurvatum*, whereas the northernmost populations of *E. minusculum* possessed haplotype H2 in common with *E. hageni*. In all three species, generally only one haplotype was present at each location; only one population in these three species contained more than two haplotypes (*E. laterale*, MA5). Overall, the genetic diversity of each species was very low ($\pi < 0.001$), and this limited amount of genetic variation did not warrant further geographical analysis.

Global levels of genetic diversity were higher in both *E. hageni* ($\pi = 0.0078$) and *E. ebrium* ($\pi = 0.0049$). The 44 haplotypes of *E. hageni* span the entire network and form 2 major groups separated by 7 mutational steps. One group is genetically diverse ($\pi = 0.0048$), whereas the other contains one common haplotype (H67) and a few mutational derivatives, resulting in a low diversity level ($\pi = 0.0008$). The 12 haplotypes found in *E. ebrium* were distributed across the network of one of the *E. hageni* groups and displayed a similar level of diversity ($\pi = 0.0049$). *E. hageni* and *E. ebrium* shared five haplotypes, of which two were very common (H35 and H41, Figs 4 and 5, Table 1). In *E. ebrium*, these 2 haplotypes accounted for 68% of the individuals, and were detected in 19 of the 25 populations surveyed, whereas they comprised 22% of the individuals and were found in 28% of the sites in *E. hageni*.

Geographical pattern of genetic variation in E. hageni *and* E. ebrium

The nested haplotype networks of *E. hageni* and *E. ebrium* are presented in Fig. 5A and 5B, respectively. The same clade numbering is used for both species to ease comparison. The network of *E. hageni* was well developed, reaching four levels of nesting and included several clades that exclusively contained that species (e.g. clades 2-2, 2-4, 2-9). The extent of the *E. ebrium* network was similar to that of the principal *E. hageni* group (clade 4-1), but *E. ebrium* haplotypes were concentrated at the tips of the network, resulting in only two levels of nesting. Many of the unsampled interior haplotypes of this network were documented in *E. hageni*, and none of the *E. ebrium* clades were exclusive to that species.

NCA results are presented in Tables 2 and 3, and the events inferred for corresponding clades in *E. hageni* and *E. ebrium* are summarized in Table 4. These analyses revealed restricted gene flow with isolation by distance

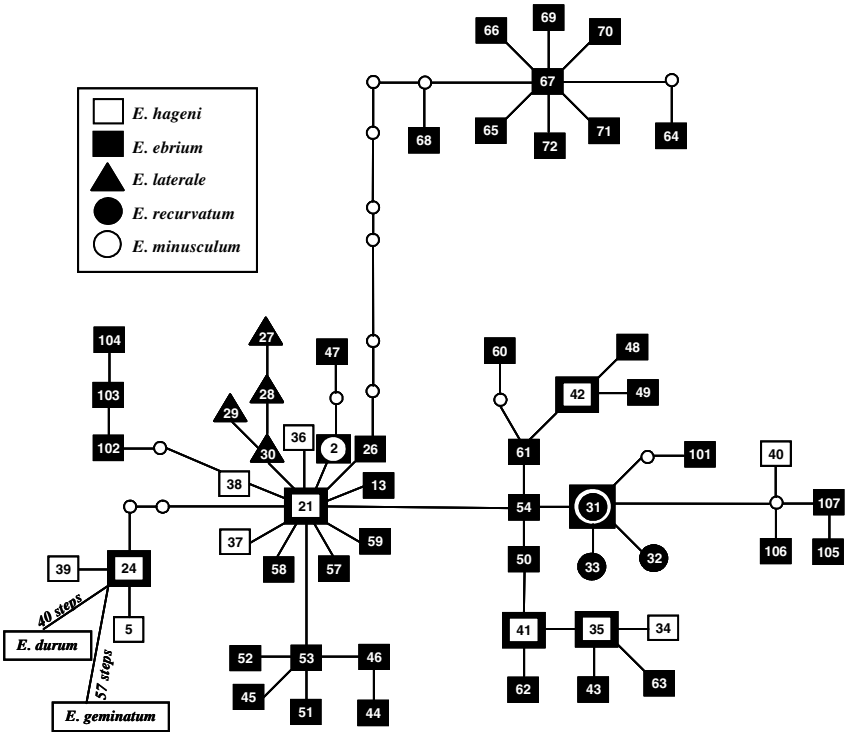

Fig. 4 Haplotype network based on the mutational differences among COI–COII mtDNA sequences found in five *Enallagma* species. Each bar represents a mutation event, whereas empty circles refer to unsampled haplotypes. Sequence codes are those of Fig. 2.

(IBD), contiguous range expansion (CRE) and past fragmentation (PF). In *E. hageni*, the most striking result was a signal of past fragmentation between clades 4-1 and 4-2. Indeed, these two groups of haplotypes are separated by seven mutations and have geographical ranges that are almost totally nonoverlapping. Clade 4-1 is not genetically very diverse, and has a very small range restricted to the southeasternmost corner of our sampling area. Clade 4-2, in contrast, spans almost all of the species' continental range. These two groups, hereafter referred to as 'Continental *hageni*' (clade 4-1) and 'Atlantic *hageni*' (clade 4-2), were found in sympatry at a single northern Vermont lake (VT19).

At lower nesting levels, restricted gene flow and IBD were the principal inferred signal. In 'Continental *hageni*', however, the clade distance of interior haplotypes were significantly small in many step-2 clades, suggesting range expansions. In clades 2-4, the expansion was most likely eastward, whereas the direction of expansion in clade 2-1 is less certain. Interior clade 1-1 has a vast range whose centre of distribution lies in the Midwest, while tip clades laid far to the east and west. The extreme locations of these tip clades suggest long-distance migration in both directions from a mid-continental origin. A very strong signal of contiguous range expansion was detected for *E. hageni* in clade 2-7, as indicated by the much wider geographical distribution of the tip clade (1-15) (D_c = 293, significantly large) relative to the interior clade (1-14: D_c = 76, significantly small; $D_{cl\text{-}T}$ = –216, significantly small, Table 2).

NCA analysis of the *E. ebrium* network yielded fewer significant results. As with *E. hageni*, a range expansion was detected in clade 2-1 for *E. ebrium*, which contains only western sites (BC, AB, ID) in this species. Here, the signal of expansion is more clearly westward. In clade 2-7, results were totally reversed from those observed in *E. hageni* (Table 4). The geographical distribution of tip clade (1-15) haplotypes in *E. ebrium* is significantly small (D_c = 241) relative to that of the interior clade 1-14 ($D_{cl\text{-}T}$ = 352, significantly large, Table 3), thus indicating restricted gene flow instead of a range expansion for a similar set of haplotypes that are extensively shared by the two species. Our sampling, which lacked sites between Idaho and Wisconsin, does not allow us to discriminate among CRE, IBD and fragmentation at the level of the entire cladogram.

Discussion

As suggested previously, all species were genetically very closely related, with mean divergence among haplotypes estimated at 0.63%. The traditional phylogenetic analysis, which does not allow for assignment of sampled haplotypes to internal nodes, could only reveal the deeper divergence between Atlantic and Continental *hageni* and, to a lesser degree, most tip clades revealed by NCA. Relationships among haplotypes were much better resolved with the network approach, as more than a third of them proved to be at internal positions. The structure of this network strongly indicates that *Enallagma hageni* represents the ancestral species for this group, and, more specifically, that all other species are derived from the Continental *hageni* stock. However, none of these species

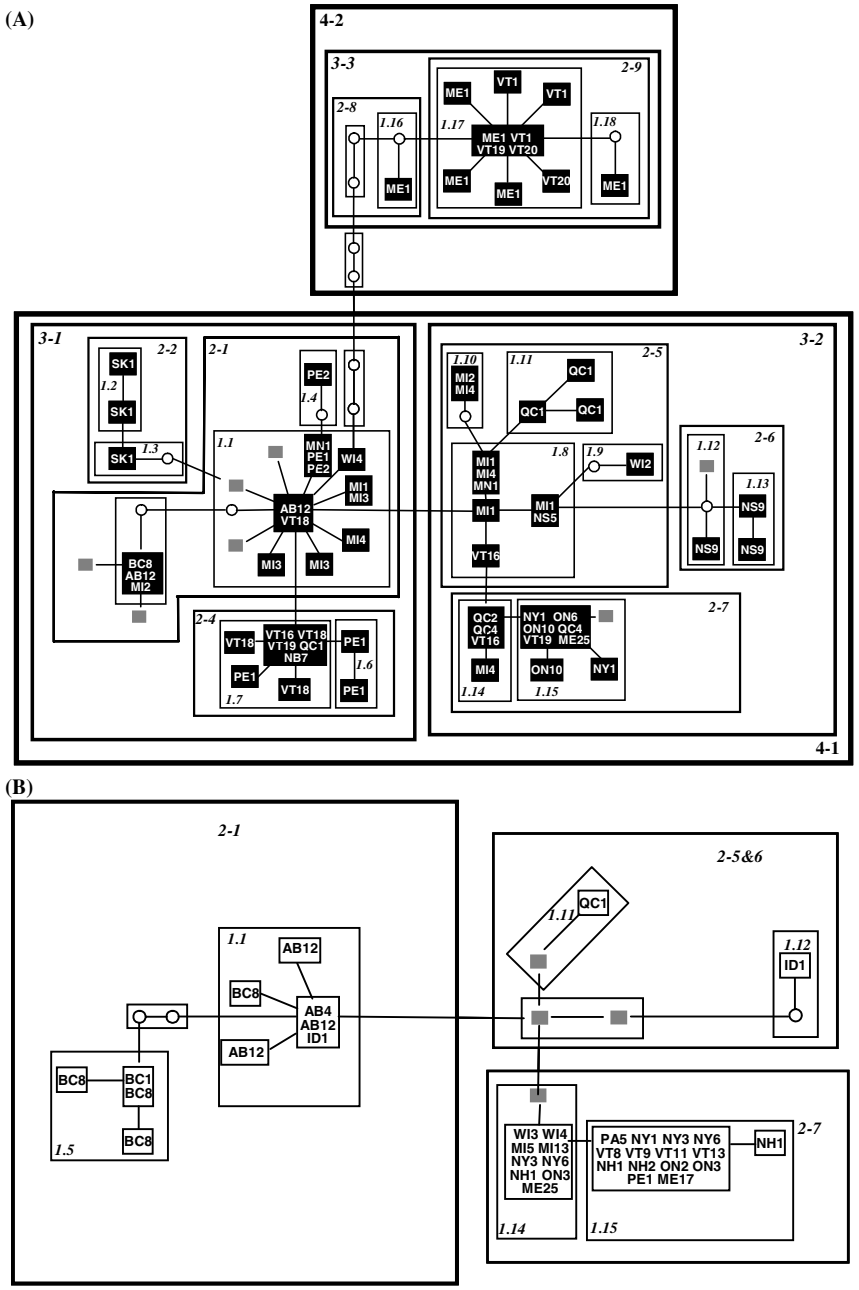

Fig. 5 COI–COII haplotype networks with structure identical to Fig. 4, but showing geographical location of haplotypes documented in (A) *Enallagma hageni* and (B) *E. ebrium*. Empty circles refer to unsampled haplotypes, whereas grey boxes are haplotypes found in (A) *E. ebrium* and (B) *E. hageni*. Nesting design utilized in NCA is shown, along with corresponding numeral clade identification for both species.

was strictly genetically diagnosable, although one species was characterized by a unique set of haplotypes (*E. laterale*). Indeed, all other species included in this analysis shared haplotypes with *E. hageni*, often when sympatric or nearly sympatric. As might be expected, lineage sorting is probably incomplete among these recently derived species (Neigel & Avise 1986), and/or they may result from multiple splitting in the ancestral *hageni* stock (species swarm). Moreover, because of the use of a maternally inherited marker, our data cannot reject the alternative hypothesis of recurrent hybridization among these species. Nevertheless, the comparative phylogeographical analysis of *E. hageni* and *E. ebrium* reveals marked contrasts in the amount and geographical distribution of genetic variation in these two species, and rather suggests distinct evolutionary histories involving different migration regimes and several recent speciation events.

The most striking result is the presence of two genetic lineages within *E. hageni*. The larger than average number of mutational steps between these lineages, as well as their almost nonoverlapping ranges, strongly supports an event of past fragmentation. Using a typical value for divergence rate of mitochondrial genes in insects of 2.35%/Myr (Brower 1994; Juan *et al*. 1995), we estimate that these two

Table 2 Nested clade analysis of *Enallagma hageni* (nesting design shown in Fig. 5A). D_c and D_n refer to average clade and nested clade distances respectively; S and L indicate significantly small and large distances respectively; i and t indicate tip and interior clade status, respectively

Haplotypes			step-1			step-2			step-3			step-4		
	D_c	D_n		D_c	D_n		D_c	D_n		D_c	D_n		D_c	D_n
2 t	1070	1173												
13 t	24	151 S												
21 i	1137	1820 L												
26 t	0	374												
57 t	0	168												
58 t	0	168 S	1-1 i	883 S	905 S									
59 t	0 S	534												
I-T	944	955 L				2-1 i	1098 S	1173						
47	-	-	1-4 t	0	2188									
24 i	0	127	1-5	1031	1556									
			I-T	110	-809 S									
103,104 i	-	-	1-2	-	-									
						2-2 t	0 S	1716						
102	-	-	1-3	-	-				3-1 i	1227 L	1252 L			
44,46 i	-	-	1-6 t	0	360 L									
45 t	0	423				2-4 t	297 S	1296						
51 t	0	212 S				I-T	850 L	-192						
52 t	0	212 S	1-7 i	255 S	263 S									
53 i	246	246	I-T	255	-98 S									
IT	246	-36												
31 t	806	1018										4-1 i	975	959
50 t	0	261												
54 i	0	825	1-8	921 L	917 L									
61 t	304	975												
IT	-529	-111												
101	-	-	1-9	0	957	2-5 i	814 L	864 L						
60	-	-	1-10	131	484									
42	-	-												
48	-	-	1-11	0 S	563				3-2 t	583 S	711 S			
49	-	-	I-T	884 L	320				I-T	644 L	541 L			
106	-	-	1-12	-	-									
105,107	-	-	1-13	-	-	2-6 t	0	913						
62 t	0	1150												
41 i	33 S	49	1-14 i	76 S	157 S									
I-T	33 S	1101												
35 i	299	301				2-7 t	292 S	329 S						
43 t	0	343				IT	530 L	518 L						
63 t	0	185	1-15 t	293 L	297 L									
I-T	299	76	I-T	-216 S	-139 S									
68	-	-	1-16	-	-	2-8	0	83						
65 t	0	86 L												
66 t	0	86 L							3-3	= = =>		4-2 t	58 S	630 S
67 i	54	56										I-T	917 L	329 L
69 t	0	77	1-17	57	57									
70 t	0	77				2-9	58	58						
71 t	0	25 S				I-T	-57	25						
72 t	0	86 L												
I-T	54	-9												
64	-	-	1-18	0	85									
			I-T	57	-28									

Table 3 Nested clade analysis of *Enallagma ebrium* (nesting design shown in Fig. 5B). D_c and D_n refer to average clade and nested clade distances, respectively; S and L indicate significantly small and large distances respectively; i and t indicate tip and interior clade status, respectively

Haplotypes			step-1				step-2			
	D_c	D_n			D_c	D_n			D_c	D_n
21 i	1137	1060								
36 t	0	579								
37 t	0	579	1-1	141 **S**	280					
38 t	0	932								
I-T	1137	363					2-1 t	263 **S**	2482 **L**	
24 i	0	127								
5 t	143	143	1-5	151 **S**	252					
39 t	0	160	I-T	-9	28					
I-T	143	0								
42	-	-	1-11 t	0	1103					
							2-5 i	1457	1457	
40	-	-	1-12 t	0	2164			-	-	
			I-T	-	-					
41	-	-	1-14 i	589	700 **L**					
35 i	240	241					2-7 t	466 **S**	803 **S**	
34 t	0	120	1-15 t	236 **S**	343 **S**		IT	1040 **L**	228	
I-T	240	116	IT	352 **L**	357 **L**					

groups split ≈ 350 000 years ago, the date given as the maximum estimate for the onset of rapid lineage diversification in this and one other clade of *Enallagma* damselfly in northeastern North America in a previous analysis of a very limited dataset (Brown *et al*. 2000). This period also roughly coincides with the end of a Pleistocene glaciation (Kansan), suggesting that fragmentation was linked to the changing climate. Clade 4-1 may then be viewed as a distinct glacial race having dispersed from an Atlantic glacial refuge whose exact location cannot be determined. One possibility is that it originated from the Georges Bank Refuge, which gave access to two major freshwater glacial lakes (Hartford and Winnipesaukee) located in the extant Connecticut and Merrimack river valleys (Larson 1980), an area roughly corresponding to the observed range of this *E. hageni* lineage. The range of this putative Atlantic race is extremely limited, and not surprisingly, gene flow appears to have been restricted within that race. In contrast, the distribution range of Continental *hageni* extends over most of the species range. Whereas the location of the distribution centre of this continental clade suggested dispersal from the Midwest (e.g. the western Great Lakes region), the entire network was rooted through haplotypes that were more prevalent at western sites, indicating that expansion may have proceeded from a centre that was farther west than the Great Lakes. In either case, our analyses revealed several events of eastward range expansions confirming that this race has been successful at dispersing into northeastern North America.

Factors that may have and are still preventing the dispersal of the Atlantic race remains unclear, but the location of the sharp phylogeographical break to the east of its range suggest that historical events may have been determinant. The only lake we have found containing both Continental and Atlantic *hageni* (VT19) stands very near to the drainage divide between the Connecticut and the Hudson rivers. In other areas along this divide, populations of Atlantic *hageni* are separated by only a few kilometres from populations of the morphologically identical, yet genetically distinct Continental *hageni*. This drainage divide also corresponds to a major physiographic discontinuity between the Mississippian and Atlantic glacial drainage systems (e.g. reviewed in Stemberger 1995). As current river drainage boundaries should not impede the dispersal of these flying insects, this observation suggests the hypothesis that the range of Atlantic *hageni* is not restricted by dispersal opportunity and potential, but that the contact zone acted, and perhaps still acts, as a hybrid sink. As all other species appear to derive

E. hageni		*E. ebrium*	
Clade	Chain event	Clade	Chain event
1-1	1-2$_{a,c}$-3-4no → IBD		1no → inc
1-7	1-2-11-17-4no → IBD		—
1-14	1-2-11$_{b,c}$-12no → CRE		inc
1-17	1-2-11-17-4no → IBD		—
2-1	1-2-11$_{b}$-12no → CRE	2-1	1-2-11b-12no → CRE
2-4	1-2-11$_{b}$-12no → CRE		—
2-5	1-2$_{a,c}$-3-4 → IBD	2-5&6	1no → inc
2-7	1-2-11$_{a,b,c}$-12no → CRE	2-7	1-2$_{a,c}$-3-4no → IBD
3-1	1-2$_{a,c}$-3-4no → IBD		
3-2	1-2$_{a,c}$-3-4no → IBD		
4-1	1-2$_{a,c}$-3-4no → IBD	total	1-2-3-5-15-16-18no → ibd/cre/pf
Total	1-2$_{a,c}$-3-4-9no → PF		—

Table 4 Historical events inferred from nested clade analysis in *Enallagma hageni* (see Table 2) and *E. ebrium* (see Table 3), following the key of Templeton *et al*. (1995). IBD: restricted gene flow and isolation by distance; CRE: contiguous range expansion; PF: past range fragmentation; inc: inconclusive

from the Continental race of *hageni*, this observation also suggest that incompatibilities between the two recently diverged groups may have promoted divergence in mate recognition system within Continental *hageni* as a means to avoid interracial matings in this area of secondary contacts.

Few inferences could be based on the relatedness and geographical distribution of *E. ebrium* haplotypes. Moreover, extensive shared polymorphisms with *E. hageni* did not readily support the independent evolutionary trajectory of this morphological taxon. However, comparison with phylogeographical patterns identified in *E. hageni* suggest that these two species are not hybridizing, and that their genetic similarity is due to incomplete lineage sorting following very recent split(s) from Continental *hageni* stock.

Within the range of the Atlantic *hageni* (southwestern Maine to eastern Vermont), *E. ebrium* haplotypes were of the Continental *hageni* type, and both species thus possessed distantly related sets of haplotypes ($N_{ST} = 0.661$). Although complete asymmetrical hybridization cannot be rejected with this mitochondrial marker, this observation strongly suggests that both species are reproductively isolated. In contrast, both species shared the same small set of haplotypes (clade 2-7) and appeared to be genetically almost identical ($N_{ST} < 0.001$) in the area immediately surrounding the range of Atlantic *hageni*, which corresponds to the region where Continental *hageni* clade 2-7 expanded (Ontario, New York, western Vermont and northern Maine). Beyond this area, however, in Wisconsin, Michigan and Prince Edward Island, these very same *E. ebrium* haplotypes are locally sympatric or nearly sympatric with a much larger array of *E. hageni* haplotypes, which were often very distinct ($N_{ST} = 0.223$). If these two species were hybridizing, it is unlikely that these contrasting patterns would correspond to geographical areas so closely associated with significant events in *E. hageni*'s recent history.

Alternatively, areas in which both species are genetically very similar could represent sites where *E. ebrium* was locally derived from *E. hageni*, and lineage sorting is incomplete. By contrast, regions in which species possess distinct sets of haplotypes would result from the recent colonization by *E. ebrium* of areas beyond the original distribution of its progenitor *E. hageni* clade. This interpretation is certainly compatible with the higher dispersal propensity of *E. ebrium* (McPeek 1989), and our failure to detect the very recent range expansion in eastern *E. ebrium* is likely due to the limit of resolution of the nested clade analysis method with genes that do not evolve at a quick enough pace. Interestingly, both species also shared a limited set of recently derived (tip) haplotypes in the west ($N_{ST} = 0.041$), suggesting that the geographical clustering of *E. ebrium* haplotypes, which NCA identified as a potential signal of fragmentation, could be interpreted as a parallel and independent origin of *E. ebrium* from *E. hageni*. Although the polyphyletic origin of a taxon may be contentious, recent evidence for the parallel evolution of mate recognition traits in other insects (Henry *et al*. 1999; Eberhard 2001) does offer some validity to this possibility that remains an hypothesis to be tested.

The presence of a sharp phylogeographical break between putative glacial races despite the dispersal capacities of the species, the plausible speciation of *E. ebrium* in an area near this break, and the close relationship of all other species with one of the two *hageni* races, altogether support a recent nonadaptive diversification fostered by divergence of mate recognition pattern upon secondary contacts of recently diverged lineages. Indeed, whereas *Enallagma* females of this clade are practically indistinguishable, the most reliable feature discriminating the adult males of each species is the form of the superior caudal appendages (i.e. cerci) they use to form tandem pairs with females prior to copulation (Westfall & May 1996). These cerci are involved in mate selection and have been shown to define the basis of a specidic mate recognition system in *Enallagma* (Paulson 1974; Robertson & Paterson 1982). Closely related species which differ primarily in traits related to courtship behaviour (e.g. Kaneshiro 1983; Knowlton 1986), courtship songs (e.g. Henry 1985; Shaw 1996a,b; Wells & Henry 1999), or genitalia lock and key mechanism (e.g. Sota & Kubota 1998) provide indirect evidence of speciation by sexual selection (Panhuis *et al*. 2001), and theoretical and empirical evidence confirm that divergence in mate choice and recognition system can lead to rapid reproductive isolation (see reviews in Panhuis *et al*. 2001 and Turelli *et al*. 2001). Variance in cerci morphology within each of the recently radiated clade is as high as that observed across the entire genus (McPeek, unpublished), and supports the role of this sexual character in the process of diversification. If any adaptive divergence has also marked this radiation, it is at best small and remains elusive, as extensive ecological work with all these species has identified no apparent differences in their ecological requirements or aptitudes (e.g. McPeek 1995, 1999, 2000; McPeek *et al*. 1996, 2001).

Although divergence in reproductive phenotype is generally recognized as a mechanism for rapid divergence, the importance of secondary contacts between recently diverged lineages as a causal event for phenotypic divergence is much more controversial in speciation research (e.g. Coyne & Orr 1998). Our contention that the contact zone revealed by this study has played a role in the *Enallagma* radiation must certainly await results from nuclear loci on the effective genetic separation of the two races (see below). At this point, our hypothesis is only indirectly supported by the presence of a phylogeographical break near the area of greatest diversity in this clade. Indeed, whereas morphologically identical *E. hageni* races seem to remain

allopatric, a speciation event is implicated just west of the break (i.e. *E. ebrium*), and the three endemic species abut the range of the Atlantic race to the east.

Conclusions and perspectives

This study confirms that many *Enallagma* species are closely related and identifies *E. hageni* as the most likely ancestral species in the clade. Patterns of genetic relatedness indicate that the four other species considered in this study were recently derived from a sublineage of *E. hageni* that probably represents a glacial race of continental origin. Despite obvious capacity and opportunities for dispersal, a genetically distinct Atlantic race of *E. hageni* occupies an exclusive but very restricted range, which suggests that genetic incompatibilities between theses races may act as a barrier to range expansion. In spite of numerous shared polymorphisms, the comparative analysis of phylogeographical patterns revealed distinct evolutionary trajectories for the two widely distributed species. Past fragmention and range expansions of great magnitude marked the history of *E. hageni*, and contrasted levels of genetic differentiation with nearby populations of *E. ebrium* suggest multiple origins followed by major recent migration movement in the latter. These evolutionary scenarios are compatible with our data and provide testable hypotheses that are the basis for further investigation of this radiation.

Our first hypothesis, the absence or a low level of gene flow between the two distinct lineages of *E. hageni*, must first be assessed by characterizing populations from areas near and away from the mitochondrial phylogeographical break with variable nuclear markers (e.g. microsatellite, amplified fragment length polymorphism). Our second hypothesis, i.e. the multiple recent origins of *E. ebrium* from local stocks of *E. hageni* followed by extensive range expansion, could also be tested by inspecting pattern of nuclear genetic variation between population pairs from specific locales. Here, our hypothesis predicts that genetic differentiation between sympatric samples should increase in area of secondary colonization, away from the speciation site(s). Finally, if differences between races of *E. hageni* are confirmed at nuclear loci, sites with Atlantic *hageni* and *E. ebrium* will allow us to gauge levels of any recent and recurrent hybridization between these morphological taxa.

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References

Albertson RC, Markert JA, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlids fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences of the USA*, **96**, 5107–5110.

Brower AVZ (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato*, inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Sciences of the USA*, **91**, 6491–6495.

Brown JM, McPeek MA, May ML (2000) A phylogenetic perspective on habitat shifts and diversity in the North American *Enallagma* damselflies. *Systematic Biology*, **49**, 697–712.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Coyne JA, Orr HA (1998) The evolutionary genetics of speciation. *Philosophical Transactions of the Royal Society of London B*, **353**, 287–305.

Danley PD, Kocher TD (2001) Speciation in rapidly diverging systems: lessons from Lake Malawi. *Molecular Ecology*, **10**, 1075–1086.

Eberhard WG (2001) Multiple origins of major novelty: moveable abdominal lobes in male sepsid flies (Diptera: Epsidae), and the question of developmental constraints. *Evolution and Development*, **3**, 206–222.

Henry CS (1985) Sibling species, call differences and speciation in green lacewings (Neuroptera: Chrysopidae: *Chyrsoperla*). *Evolution*, **39**, 965–984.

Henry CS, Wells MLM, Simon CM (1999) Convergent evolution of courtship songs among cryptic species of the *Carnea* group of green lacewings (Neuroptera: Chrysopidae: *Chrysoperla*). *Evolution*, **53**, 1165–1179.

Hubbell SP (2001) *The Unified Neutral Theory of Biodiversity and Biogeography*. Princeton University Press, Princeton, NJ.

Johnson DM, Crowley PH (1980) Habitat and seasonal segregation among coexisting odonate larvae. *Odonatologica*, **9**, 297–308.

Juan C, Oromi P, Hewitt GM (1995) Mitochondrial DNA phylogeny and sequential colonization of Canary Islands by darkling beetles of the genus *Pimelia* (Tenebrionidae). *Proceeding of the Royal Society of London*, **261**, 173–180.

Kaneshiro KY (1983) Sexual radiation and direction of evolution in the biosystematics of Hawaiian Drosophilidae. *Annual Review of Ecology and Systematics*, **28**, 161–178.

Knowlton N (1986) Cryptic and sibling species among the decapod Crustacea. *Journal of Crustacean Biology*, **6**, 356–363.

Larson GJ (1980) Late Wisconsinan glaciation of New England. In: *Late Wisconsinan Glaciation of New England* (eds Larson GJ, Stone BD), pp. 101–114. Kendall/Hunt, Dubuque, IA.

McCann KS (2000) The diversity–stability debate. *Nature*, **405**, 228–233.

McPeek MA (1989) Differential dispersal tendencies among *Enallagma* damselflies (Odonata: Coenagrionidae) inhabiting different habitats. *Oikos*, **56**, 187–195.

McPeek MA (1990a) Determination of species composition in the *Enallagma* damselfly assemblage of permanent lakes. *Ecology*, **71**, 83–98.

McPeek MA (1990b) Behavioral differences between *Enallagma* species (Odonata) influencing differential vulnerability to predators. *Ecology*, **71**, 1714–1726.

McPeek MA (1995) Morphological evolution mediated by behavior in the damselflies of two communities. *Evolution*, **49**, 749–769.

McPeek MA (1996) Linking local species interactions to rates of speciation in communities. *Ecology*, **77**, 1355–1366.
McPeek MA (1997) Measuring phenotypic selection on an adaptation: lamellae of damselflies experiencing dragonfly predation. *Evolution*, **54**, 459–466.
McPeek MA (1998) The consequences of changing the top predator in a food web: a comparative experimental approach. *Ecological Monographs*, **68**, 1–23.
McPeek MA (1999) Biochemical evolution associated with antipredator adaptation in damselflies. *Evolution*, **53**, 1835–1845.
McPeek MA (2000) Predisposed to adapt? Clade-level differences in characters affecting swimming performance in damselflies. *Evolution*, **54**, 2072–2080.
McPeek MA, Brown JM (2000) Building a regional species pool: diversification of the *Enallagma* damselflies in eastern North America. *Ecology*, **81**, 904–920.
McPeek MA, Grace M, Richardson JML (2001) Physiological and behavioral responses to predators shape the growth/predation risk trade-off in damselflies. *Ecology*, **82**, 1535–1545.
McPeek MA, Schrot AK, Brown JM (1996) Adaptation to predators in a new community: swimming performance and predator avoidance in damselflies. *Ecology*, **77**, 617–629.
Neigel JE, Avise JC (1986) Phylogenetic relationships of mitochondrial DNA under various demographic model of speciation. In: *Evolutionary Processes and Theory* (eds Karlin S, Nevo E), pp. 515–534. Academic Press, San Diego.
Panhuis TM, Butlin R, Zuk M (2001) Sexual selection and speciation. *Trends in Ecology and Evolution*, **16**, 364–371.
Paulson DR (1974) Reproductive isolation in damselflies. *Systematic Zoology*, **23**, 40–49.
Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.
Robertson HM, Paterson HEH (1982) Mate recognition and mechanical isolation in *Enallagma* damselflies (Odonata: Coenagrionidae). *Evolution*, **36**, 243–250.
Schluter D (2000) *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.
Seehausen O, Mayhew PJ, van Alphen JJM (1999) Evolution of colour patterns in East African cichlid fish. *Journal of Evolutionary Biology*, **12**, 514–534.
Seehausen O, van Alphen JJM, Witte F (1997) Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science*, **277**, 1808–1811.
Shaw KL (1996a) Polygenic inheritance of a behavioral phenotype: interspecific genetics of song in the Hawaiian cricket genus *Laupala*. *Evolution*, **50**, 256–266.
Shaw KL (1996b) Sequential radiations and patterns of speciation in the Hawaiian cricket genus *Laupala* inferred from DNA sequences. *Evolution*, **50**, 237–255.
Sota T, Kubota K (1998) Genital lock-and-key as a selective agent against hybridization. *Evolution*, **52**, 1507–1513.
Stemberger RS (1995) Pleistocene refuge areas and postglacial dispersal of copepods of the northeastern United States. *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 2197–2210.
Swofford DL (2002) PAUP*: *Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0b10 for Windows*. Florida State University, Tallahassee, FL, USA.
Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.
Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.
Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA halotypes in the tiger Salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.
Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analysis with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.
Tilman D, Pacala S (1993) The maintenance of species richness in plant communities. In: *Species Diversity in Ecological Communities: Historical and Geographical Perspectives* (eds Ricklefs RE, Schluter D), pp. 13–25. University Press of Chicago, Chicago.
Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. *Trends in Ecology and Evolution*, **16**, 330–342.
Walker BH (1992) Biological diversity and ecological redundancy. *Conservation Biology*, **6**, 18–23.
Wells MM, Henry CS (1999) Songs, reproductive isolation, and speciation in cryptic species of insects. In: *Endless Forms: Species and Speciation* (eds Howard DJ, Berlocher SH), pp. 217–233. Oxford University, Oxford.
Westfall MJ, May ML (1996) *Damselflies of North America*. Scientific Publishers, Gainesville FL.

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