

# Solving cryptogenic histories using host and parasite molecular genetics: the resolution of *Littorina littorea*'s North American origin

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## Abstract

Even after decades of investigation using multiple sources of evidence, the natural histories of some species remain unclear (i.e. cryptogenic). A key example is *Littorina littorea*, the most abundant intertidal snail in northeastern North America. Native to Europe, the snail's ecological history in North America has been debated for over 100 years with no definitive resolution. To resolve its cryptogenic status, we used molecular genetics from a novel combination of the snail and a highly associated trematode parasite, *Cryptocotyle lingua*. Based on mitochondrial sequences of 370 *L. littorea* and 196 *C. lingua* individuals, our results demonstrate a significant reduction in genetic diversity in North America vs. Europe, North American haplotypes nested within European haplotypes, and mean divergence estimates of ~500 years ago from Europe for both host and parasite – thus supporting a recent introduction of both host and parasite to North America from Europe. Our study therefore resolves not only a specific cryptogenic history, but it also demonstrates the success of our approach generally and could be used in resolving difficult invasion histories worldwide.

**Keywords:** biogeography, *Cryptocotyle lingua*, cryptogenic, introduction, *Littorina littorea*, population divergence

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## Introduction

In recent years, invasive species have become recognized as a major component of human-mediated impacts on natural systems. However, there remain a considerable number of species that cannot be demonstrably classified as native or non-native in many regions, and these species are referred to as cryptogenic (Carlton 1996; Ruiz *et al.* 2000). Especially in the case of conspicuous, abundant, or high-impact species, resolution of the ambiguous ecological history of cryptogenic species is a critical conservation goal. One cryptogenic species, whose history in North America has vexed scientists for 150 years (Ganong 1886; Clarke & Erskine 1961; Berger 1977; Reid 1996; Wares *et al.* 2002; Chapman *et al.* 2007, 2008; Wares & Blakeslee 2007; Cunningham 2008) is the European marine snail, *Littorina littorea* (common periwinkle) (Prosobranchia: Littorinidae;

Linnaeus, 1758), a conspicuous rocky intertidal snail abundant on both North Atlantic coasts. Cryptogenic status often stems from incomplete or unknown historical knowledge of a species' presence in a location. Thus, the lingering ambiguity surrounding *L. littorea*'s status is particularly surprising given the vast amount of research over the past several decades that has been conducted on this species (e.g. Berger 1977; Wares *et al.* 2002). Considering its conspicuousness and dominance within intertidal regions in northeast North America and past use as a textbook case of invasion (e.g. Steneck & Carlton 2001), the resolution of *L. littorea*'s present-day cryptogenic status is a fundamental ecological question.

## *Equivocal evidence for Littorina littorea's North American Origin*

*Littorina littorea*'s spread into the USA from Canada in the mid-1800s and its impacts upon marine biota in this region have been well documented (e.g. Carlton 1982; Brenchley & Carlton 1983; Lubchenco 1983; Bertness 1984; Yamada & Mansour 1987); yet uncertainty has remained regarding

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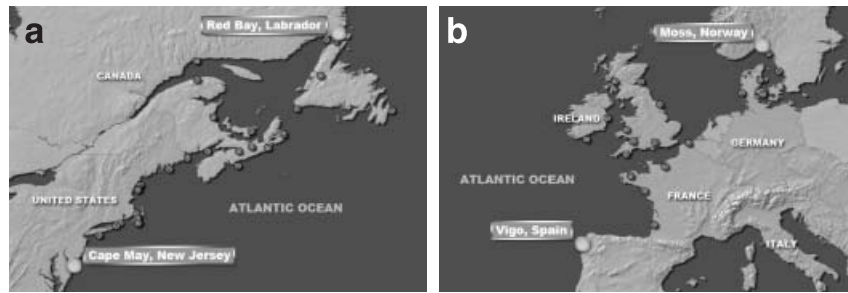
the snail's status as native or non-native in North America. The two scenarios debated are whether the Canadian population that spread into the USA was a native glacial relic confined to the Canadian Maritimes until the mid-1800s, or whether it was an anthropogenically introduced population from Europe (Reid 1996). Although evidence has been provided by several researchers since 1886 and has included historical, archaeological, ecological, and molecular data (Ganong 1886; Clarke & Erskine 1961; Bird 1968; Berger 1977; Carlton 1982; Johannesson 1992; Reid 1996; Wares *et al.* 2002; Chapman *et al.* 2007, 2008; Wares & Blakeslee 2007; Cunningham 2008), its North American cryptogenic status remains unresolved because conclusions from these sources have been conflicting or equivocal (Johannesson 1992; Reid 1996; Chapman *et al.* 2007, 2008; Wares & Blakeslee 2007; Cunningham 2008).

The evidence related to *L. littorea*'s North American origin has been reviewed in several recent publications (especially Reid 1996; Blakeslee 2007; and Chapman *et al.* 2007, 2008); here, we will briefly summarize these findings. First, historical sources of evidence have included conflicting oral testimonies of its presence in the Canadian Maritimes prior to the 1850s (Verrill 1874; Ganong 1886; Reid 1996). In addition, prior to 1870 *L. littorea* was never described in any North American naturalist/field records, while less conspicuous snails, like *Littorina obtusata*, *Littorina saxatilis* and *Ilyanassa obsoleta*, were frequently found in published reports (e.g. Gould 1852; Binney 1863). Second, the archaeological evidence has also been equivocal. A handful of *L. littorea* specimens have been found in archaeological sites in maritime Canada; however, only a subset have been radiocarbon-dated and of these, none have predated early European and Norse visits to the maritime region (Chapman *et al.* 2007). Third, ecological sources of evidence/hypotheses for its North American presence have included the suggestion that *L. littorea* could have rafted on driftwood/seaweed for a natural invasion of North America from Europe (Bird 1968); however, *L. littorea* is not found in Iceland or Greenland (Johannesson 1988; Reid 1996), likely ruling out stepping-stone invasion across islands in the North Atlantic, and a direct crossing of drifting adults would be expected to end up south of the snail's current North American range (Kraeuter 1976). Moreover, *L. littorea* uses planktotrophic dispersal of larvae, and a direct crossing of larvae is believed highly improbable, if not impossible (Kraeuter 1976; Reid 1996). Further ecological evidence has involved the well-supported theory of enemy escape (Torchin *et al.* 2003), in which *L. littorea* was found to have a significantly lower trematode parasite species richness in *L. littorea* in North America compared to Europe — evidence which appears consistent with a recent introduction of the snail to North America (Blakeslee & Byers 2008). Lastly, a variety of molecular techniques have been used to understand *L. littorea*'s ecological history in North America, from

allozyme analyses in the 1970s (Berger 1977; Morris 1979) to DNA sequencing (Wares *et al.* 2002) and amplified fragment length polymorphisms (AFLPs) (Wares & Blakeslee 2007) in recent times [this considerable evidence has been summarized and analysed extensively in both Chapman *et al.* (2007) and Blakeslee (2007)]. One study in particular (Wares *et al.* 2002), which included the greatest number of samples prior to our investigation, concluded that *L. littorea* was native to North America based on their DNA sequence data. However, recent studies (Chapman *et al.* 2007; Wares & Blakeslee 2007) have questioned the study's conclusions based on sample size issues, particularly regarding calculated divergence estimates and the assumption that unshared North American haplotypes represented endemism. Because no molecular study has yet been able to include enough information to conclusively answer the debate over *L. littorea*'s North American origin, several researchers have called for a larger molecular data set to definitively resolve *L. littorea*'s cryptogenic status in north-eastern North America (Reid 1996; Wares & Blakeslee 2007; Chapman *et al.* 2008; Cunningham 2008).

#### *Multiple lines of evidence to resolve L. littorea's cryptogenic status*

In our investigation, we set out to improve upon these prior limitations in the molecular work by heavily sampling the snail over a wide geographical range in both the European and North American populations. The mitochondrial data set we compiled is almost four times the size of the previously largest data set, and as we demonstrate here, our overall larger sample size allows us to produce robust estimates of population divergence. In addition, we recognize that due to the equivocal nature of *L. littorea*'s North American presence, multiple lines of evidence are necessary to fully resolve its cryptogenic history. In particular, we include here not only a molecular genetic analysis of the snail itself (Fig. 1) but additionally a broad molecular sampling of an associated, host-specific trematode parasite, *Cryptocotyle lingua* (Digenea: Heterophyidae; Creplin, 1825) — *L. littorea*'s most common parasite in both North America and Europe, and thus the most likely candidate for an associated introduction with the snail (Byers *et al.* 2008; Blakeslee & Byers 2008). Because parasites have recently become recognized as important tools/indicators in the understanding of host source populations (Criscione *et al.* 2006), we felt that the inclusion of such a corroborative data set along with evidence from the snail itself could help definitively resolve *L. littorea*'s cryptogenic status in North America. Finally, our complementary approach of host and parasite molecular genetics is important since our molecular data set for *L. littorea* includes one loci, and a recent AFLP data set (Wares & Blakeslee 2007) exploring multiple loci within the snail was unable to



**Fig. 1** North American (a) and European (b) collection sites for *Littorina littorea* snails and *Cryptocotyle lingua* trematodes. For *L. littorea*, there were a total of 29 North American sample sites, ranging from Red Bay, Labrador to Cape May, New Jersey, and 22 European sample sites, ranging from Moss, Norway to Vigo, Spain. *C. lingua* infections were found at a subset of *L. littorea* sites: there were 20 North American sample sites, ranging from Red Bay, Labrador to Point Judith, Rhode Island, and 16 European sample sites, ranging from Moss, Norway to Mindin, France. See Appendices I and II for site locations.

effectively provide any further resolution. Thus, novel information is truly needed to definitively resolve this century-old question.

In both *L. littorea* and *C. lingua*, we tested whether the snail and trematode exhibited molecular signatures expected of a recent founder event, which would include lower genetic diversity in North America vs. Europe, distributions of North American haplotypes nested within European haplotypes (Grosberg & Cunningham 2000), and short divergence time estimates between the North American and European populations because coalescent theory would predict a recent split between the two regions. However, if the alternative hypothesis were true — that *L. littorea* were native to North America having existed historically and through the most recent Ice Age in refugia in maritime Canada until its spread into the USA in the mid-1800s — we expected population divergence estimates to be prior to human contact with North America from Europe.

## Materials and methods

### Collections and molecular processing

Snails were collected from 29 North American ( $n = 183$ ) and 22 European ( $n = 187$ ) sites (Fig. 1; Appendix I). Snails were dissected to preserve foot tissue for DNA analyses and also to look for parasitism by *Cryptocotyle lingua*. Only uninfected snails (as a conservative precaution to avoid potential contamination issues) were used in host molecular analyses. *Cryptocotyle lingua* parasites were extracted from gonadal tissues of infected snails and preserved for molecular analyses. This trematode is easily distinguished from all other trematodes infecting *Littorina littorea* in that it is the only cercaria with two eyespots (James 1968). *Cryptocotyle lingua* parasites were found at 20 North American ( $n = 98$ ) and 16 European ( $n = 98$ ) sites (Fig. 1; Appendix II). All DNA was extracted using a standard CTAB protocol (France *et al.* 1996). In the snail, two sets of primers amplified cytochrome

*b* (cyt *b*) and cytochrome oxidase I (COI) mitochondrial genes: cyt *b* (625 bp): *Primer1-F*, CCTTCCCGCACCTTCAAATC; *Primer4-R*, ATGAGAAATTTTCAGGGTC (Reid *et al.* 1996); COI (572 bp): *LLCOIAB-F*, CTCTCCTGGGAGATGACCAG; *LLCOIAB-R*: TTCTGGGTGACCGAAGAATC designed using prior sequence data (Williams & Reid 2004). Snail samples were amplified using an adapted polymerase chain reaction (PCR) protocol (Kyle & Boulding 1998) and subjected to 32 cycles of 95 °C for 30 s, 44 °C for 30 s, and 72 °C for 30 s. For the trematode, two sets of primers amplified two contiguous regions (1043 bp) of the COI mitochondrial gene: *COI2575F*: TTTTTGGGCATCCTGAGGTTTAT; *COI3021R*: TAAAGAAAGAACA-TAATGAAAATG (Morgan & Blair 1998); *ABCOICLF*: TCTTTAGGATCATAAGCG; *ABCOICLR*: TAAACCCCGTATCCAAACC designed using prior COI sequence data (Kane 1999). Trematode samples were amplified using an adapted PCR protocol (Huspeni 2000) subjected to 35 cycles of 94 °C for 30 s, 50.9 °C for 30 s, and 72 °C for 30 s. Following PCR, samples were cleaned-up for sequencing using a Qiaquick QIAGEN Kit. When samples were sequenced, each sample was run in both the forward and reverse directions and then later aligned in order to ensure haplotype identities were accurately assigned. Sequences were aligned using DNASTar Lasergene programs.

### Statistical analyses

Phylogenetic relationships were analysed using PAUP\* 4.0 (Swofford 2003). Phylogenetic trees were not only constructed using the full data set, but also using a truncated data set, where we excluded all third position sites (resulting in 798 total bp for *L. littorea* and 695 bp for *C. lingua*), which are the most variable sites in coding DNA because substitutions at these sites are often silent. We surmised that this latter approach would give us a conservative estimate of haplotype diversity in Europe vs. North America. Finally, in *L. littorea* we constructed a

phylogenetic tree of just North American individuals so we could compare Canadian vs. US sites. We performed this last analysis as a way to determine whether Canadian sites showed more diversity than US sites, which might be expected if *L. littorea* had existed in the Canadian Maritimes for thousands or hundreds of thousands of years before spreading into the USA ~150 years ago. For each of these phylogenetic analyses, the maximum likelihood root haplotype for each tree (designated by an asterisk) was determined using the program, rcs 1.21 (Clement *et al.* 2000).

Because haplotype diversity was high in our sampled populations, we used haplotype estimation curves to estimate haplotype diversity in each population and to quantify the effects of sampling effort on haplotype diversity. Specifically, we used ESTIMATES 8.0 (Colwell 2006) to calculate haplotype accumulation and haplotype estimation curves. ESTIMATES uses Monte Carlo resampling [through randomization of sample order over a number of replicates (e.g. 500)] to determine the mean accumulation of haplotypes ( $S_{obs}$ ) as samples are added over the full data set, while also providing standard deviations and 95% confidence intervals for each data point (Gotelli & Colwell 2001). However, sample-based rarefaction curves may not capture the entire haplotype diversity within a population for a particular sampling effort, especially if these curves have not reached a stable asymptote (Gotelli & Colwell 2001). Thus, nonparametric estimators, such as Chao2, can be useful in predicting the eventual asymptote in haplotype diversity for a particular population and do so by including the effects of rare haplotypes on the total haplotype diversity (Gotelli & Colwell 2001). The Chao2 estimator has been found to be one of the most robust estimators (see Chao2 equation in Colwell 2006) when compared to empirical data from a variety of systems (Walther & Morand 1998; Foggo *et al.* 2003).

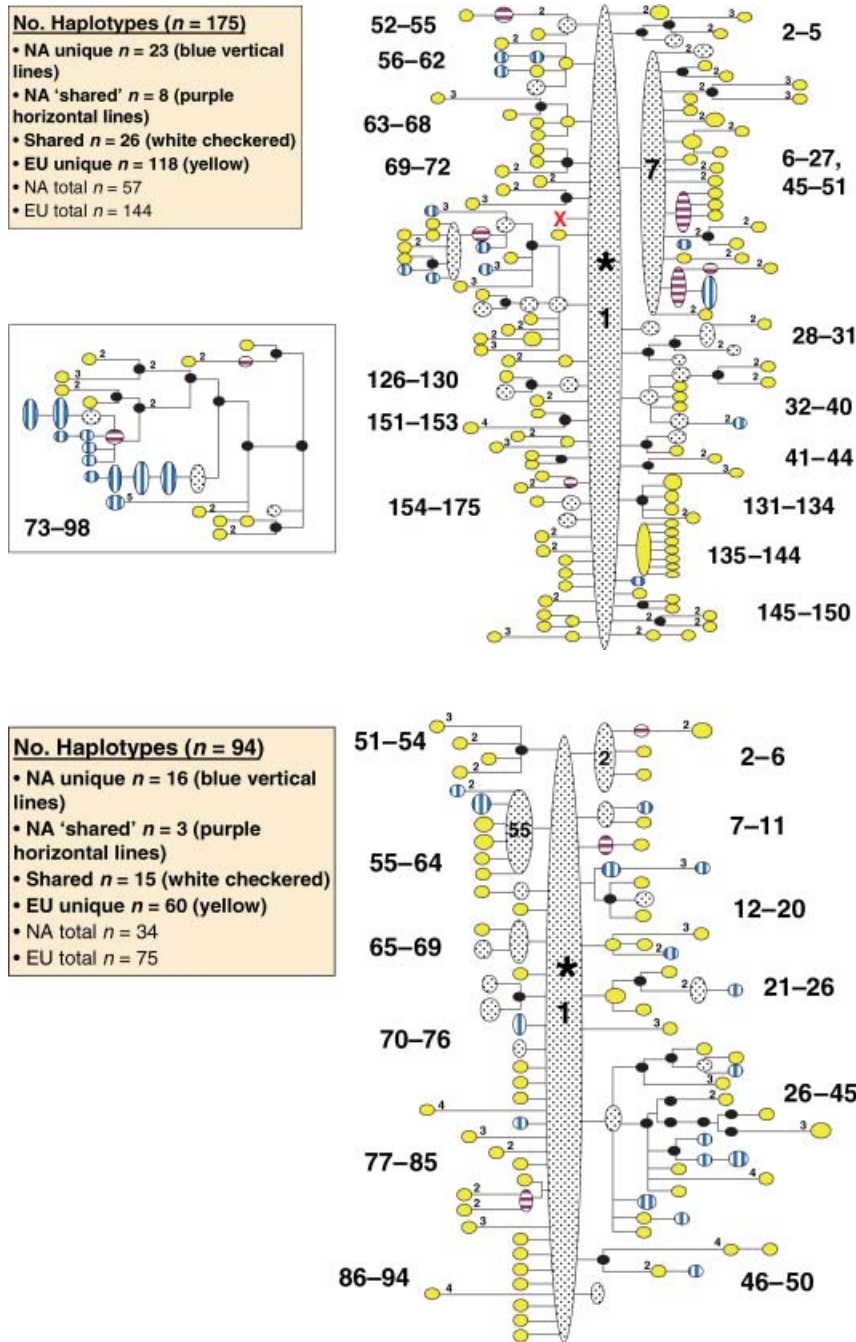
Finally, population divergence estimates were performed using the (isolation with migration) IM program (July 2006 version; Hey & Nielsen 2004, 2007). IM uses Markov chain Monte Carlo sampling and applies the isolation with migration model to genetic data taken from closely related species or populations of the same species. The program provides maximum-likelihood estimates of the time since two populations split ( $t$ ) in terms of mutations, which can be converted to an estimate of the number of years since the populations diverged using the specific mutation rate for the gene in question. We performed 10 different runs/replicates of IM for each of the snail and trematode sequences using the following input parameters and 10 different random seeds:  $q1 = 5000$ ,  $m1 = m2 = 7$ ,  $t = 2$ ,  $b = 100\ 000$ . Divergence estimates were calculated using the following equation:  $t = t/\mu$ , where  $t$  is the total years of divergence time,  $t$  is the time parameter determined by IM from the sequence data, and  $\mu$  is the gene substitution rate

(Hey & Nielsen 2004). The substitution rate [3% per million years (Myr);  $\sim 1.8 \times 10^{-5}$  for 1197 bp] that we used in calculating divergence estimates for *L. littorea* was determined from fossil record evidence of *Littorina* sp. (Reid *et al.* 1996; Wares & Cunningham 2001; and employed in Wares *et al.* 2002; Chapman *et al.* 2008; Cunningham 2008). However, we also included a range of mutation rates (2–4% per Myr) for comparison since a single mutation rate based on fossil evidence is still an estimate of substitution; thus, a range of mutation rates provide a more robust understanding of divergence time between the populations. Because trematodes do not preserve well in the fossil record, the best estimates of COI substitution rates for trematodes, like *C. lingua*, is a range between 2% and 4% per Myr (J.A.T. Morgan, personal communication): for 1043 bp, the rates used in calculating divergence estimates were therefore:  $1.04 \times 10^{-5}$  for 2% per Myr,  $1.56 \times 10^{-5}$  for 3% per Myr, and  $2.09 \times 10^{-5}$  for 4% per Myr.

## Results

We found *Littorina littorea* to possess a total of 175 haplotypes (BLAST Accession nos EU875593–EU876332) from 370 total sequences ( $n = 187$  in Europe and  $n = 183$  in North America) over a total of 1197 base pairs (Fig. 2; Appendix I). Fifty-seven haplotypes were from North America, and 144 were from Europe (these numbers include shared haplotypes). Altogether, North America showed a significant reduction in genetic diversity compared to Europe ( $\chi^2 = 37.7$ , d.f. = 1,  $P < 0.001$ ). The majority of European haplotypes in *L. littorea* were only observed once (89% were rare, 11% common), while in North America many more haplotypes were observed more than once (58% rare, 42% common). Furthermore, no clades were completely monophyletic for North American individuals (Fig. 2). To predict expected haplotype totals in each population, we performed haplotype estimation (Chao2) analyses and found the expected, maximum number of haplotypes in Europe to be 2456 (95% CI, 918–4115) compared to the 144 observed in Europe. In North America, 140 haplotypes (95% CI, 89–273) were predicted compared to the 57 that were observed (Fig. 4); therefore, haplotype diversity in Europe was estimated by the Chao2 analysis to be 17.5 times greater than in North America.

For *Cryptocotyle lingua*, we found similar patterns to *L. littorea* in haplotype identities and frequencies: a total of 94 haplotypes (BLAST Accession nos EU876333–EU876528) were found from 196 sequences ( $n = 98$  in both Europe and North America) over a total of 1043 base pairs (Fig. 3; Appendix II). Thirty-four haplotypes were North American and 75 were European (these numbers include shared haplotypes), and North America was significantly reduced in genetic diversity compared to Europe ( $\chi^2 = 10.78$ , d.f. = 1,  $P < 0.001$ ). The majority of European haplotypes in *C. lingua* were only observed once (88% were rare, 12% common),



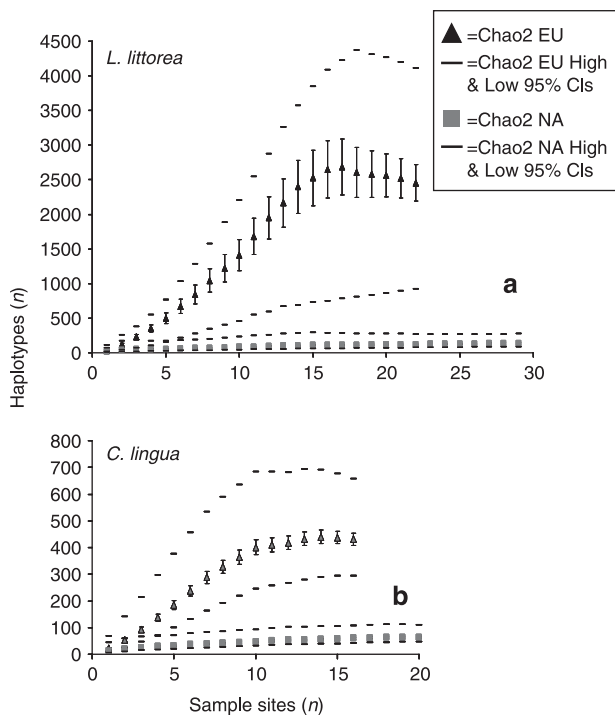
**Fig. 2** Haplotype tree for *Littorina littorea*. Numbering represents haplotype identities within clades on the tree (see Appendix I). Haplotype bubbles are relatively sized based on haplotype frequencies and are coloured according to the following categories: unique to Europe (yellow), unique to North America (blue vertical lines), shared between populations (white chequered), and a fourth category (purple horizontal lines) for North American haplotypes basal to European haplotypes (and thus considered shared). The inset represents a clade that was too large for the scale of this diagram (see the 'X' for position on the overall tree). The haplotype with an asterisk represents the maximum likelihood root. The small numbers above lines connecting haplotype bubbles represent the number of mutations that have occurred since the prior haplotype to the haplotype in question. The bold numbers down each side of the figure refer to haplotype identities found in Appendix I.

**Fig. 3** Haplotype tree for *Cryptocotyle lingua*. Numbering represents haplotype identities within clades on the tree (see Appendix II). Haplotype bubbles are relatively sized based on haplotype frequencies and are coloured according to the following categories: unique to Europe (yellow), unique to North America (blue vertical lines), shared between populations (white chequered), and a fourth category (purple horizontal lines) for North American haplotypes basal to European haplotypes (and thus considered shared). The haplotype with an asterisk represents the maximum likelihood root. The small numbers above lines connecting haplotype bubbles represent the number of mutations that have occurred since the prior haplotype to the haplotype in question. The bold numbers down each side of the figure refer to haplotype identities found in Appendix I.

while in North America many more haplotypes were observed more than once (53% rare, 47% common). Again, no clades were completely monophyletic for North American individuals (Fig. 3). Haplotype estimates (Chao2) for the expected, maximum number of haplotypes were found to be 430 (95% CI, 293–656) in Europe vs. 64 (95% CI, 46–110) in North America (Fig. 4).

Phylogenetic comparisons within the North American region for *L. littorea* revealed diversity between the two populations that was essentially equal (Canada: 29 total

haplotypes from 95 individuals, and USA: 28 total haplotypes from 88 individuals) and not significantly different ( $\chi^2 = 0.2$ , d.f. = 1,  $P = 0.895$ ). Additionally, the number of unique Canadian haplotypes was nearly identical to the number of unique US haplotypes (19 and 18, respectively), and 10 haplotypes were shared between the two regions. Chao2 estimates of haplotype diversity were 82 (95% CI, 58–139) in Canada vs. 70 (95% CI, 60–117) in the USA, suggesting similar maximum estimates between the two regions.



**Fig. 4** Haplotype estimation curves for European (EU) vs. North American (NA) *Littorina littorea* (a) and *Cryptocotyle lingua* (b). The European Chao2 estimator suggests a maximum, expected number of haplotypes of ~2500 (with 95% CIs of ~920 and ~4110 haplotypes) for *L. littorea* and 430 (with 95% CIs of ~290 and ~650 haplotypes) for *C. lingua*, while the North American Chao2 estimator suggests a maximum, expected number of haplotypes of ~140 (with 95% CIs of ~90 and ~270 haplotypes) for *L. littorea* and 64 (with 95% CIs of ~45 and ~110 haplotypes) for *C. lingua*. These data demonstrate the much greater genetic diversity expected in Europe compared to North America, which strongly suggests that the 23 and 16 unique haplotypes found in North American *L. littorea* and *C. lingua*, respectively, are likely among the multiple haplotypes yet to be found in Europe (error bars are standard error).

Finally, divergence estimates from the snail and trematode sequence data for Europe and North America were calculated using the *IM* program (Hey & Nielsen 2004, 2007), which is well suited for asking questions about recent invasions since it does not include assumptions for equal effective population sizes between populations, which is known to have been problematic for prior divergence estimates using *L. littorea* sequence data (Chapman *et al.* 2007; Cunningham 2008). Over 10 replicate runs for *L. littorea*, we found mean ( $\pm$  SE) divergence estimates for the three mutation rates to be: 668 ( $\pm$  132) years ago with 95% CIs between 518 ( $\pm$  109) and 969 ( $\pm$  205) years ago for the 2% per Myr mutation rate; 444 ( $\pm$  88) years ago with 95% CIs between 344 ( $\pm$  73) and 644 ( $\pm$  137) years ago for the 3% per Myr mutation rate [the accepted mutation rate for littorine snails based upon molecular and fossil record evidence

(Reid *et al.* 1996; Wares & Cunningham 2001; and employed in Wares *et al.* 2002; Cunningham 2008; Chapman *et al.* 2008)]; and 334 ( $\pm$  66) years ago with 95% CIs between 259 ( $\pm$  55) and 485 ( $\pm$  102) years ago for the 4% per Myr mutation rate. Based on the uncertainty for mutation rates in trematodes, we also conducted *IM* over a range of mutation rates (2–4% per Myr) for *C. lingua*; all produced highly similar divergence estimates. We found the mean ( $\pm$  SE) divergence estimates to be: 460 ( $\pm$  160) years ago with 95% CIs between 306 ( $\pm$  123) and 690 ( $\pm$  229) years ago for the 2% per Myr mutation rate; 306 ( $\pm$  114) years ago with CIs between 204 ( $\pm$  82) and 460 ( $\pm$  153) years ago for the 3% per Myr mutation rate; and 230 ( $\pm$  85) years ago with CIs between 153 ( $\pm$  61) and 345 ( $\pm$  115) years ago for the 4% per Myr mutation rate.

## Discussion

Our results demonstrate several genetic signatures that strongly support a founder effect in North American *Littorina littorea* and its associated trematode *Cryptocotyle lingua*. First, both the snail and trematode showed significant reductions in overall genetic diversity in North America compared to Europe for both the complete data sets (Figs 2 and 3) and in a truncated data set where we excluded all third position sites, thus presenting a conservative estimate of genetic diversity in each population. In the latter data set, we found all but two North American *L. littorea* haplotypes and all but four *C. lingua* haplotypes to be nested within European haplotypes. Given the significant diversity in Europe, the fact that these two to four haplotypes were not nested within European haplotypes is likely a product of under-sampling in Europe and not North American endemism (see detailed discussion below regarding unshared haplotypes in North America).

Additionally, no clades for either the snail or the trematode were completely monophyletic for North American individuals (Figs 2 and 3). This demonstrates that not a single North American individual sequence or haplotype was completely independent from Europe, suggesting that further sampling should reveal shared status and thus nestedness of all North American genetic diversity within European diversity.

We found some seemingly unique haplotypes in North America (23 unique North American haplotypes in *L. littorea* and 16 in *C. lingua*). These findings are most likely the result of under-sampling (i.e. these unshared haplotypes went undetected in the native range) rather than representing endemism to North America. Our haplotype estimation curves support this interpretation because they predict European diversity in *L. littorea* to be over an order of magnitude greater than North American diversity, requiring significantly more sampling in Europe to reveal all predicted haplotypes (Fig. 4). Thus, the 23 unshared North

American haplotypes are likely among the 2000+ haplotypes that have yet to be discovered in Europe — a pattern consistent for the trematode as well. Due to practical limits on sampling effort, it is not uncommon even in investigations of definitively known species introductions to detect unique haplotypes in the founding populations that are not observed in samples from the source populations (Miura *et al.* 2006; Roman 2006; among others).

Divergence estimates also support a recent founding event for North American *L. littorea* and its associated trematode, *C. lingua*, since all estimates were within the time frame for human colonization of North America from Europe. *L. littorea*'s first reported sighting was in Pictou, Nova Scotia, settled by Europeans in the mid-1600s; however, Vikings are also believed to have visited maritime Canada as far back as ~1000 years ago (Spjeldnaes & Henningsmoen 1963). Even with a range of mutation rates, all of our estimates suggested a divergence between Europe and North America of less than 1000 years ago (when including 95% CI estimates) with mean estimates for the three mutation rates ranging between 334 years and 668 years ago for *L. littorea* and between 230 years and 460 years ago for *C. lingua*. We can have confidence that our greater sampling effort has produced robust divergence estimates for both the snail and trematode given our tight 95% CIs for all estimates [which were on the order of tens to hundreds of years different from the mean estimate as opposed to prior divergence estimates where CIs were sometimes thousands to tens of thousands of years different from the mean (e.g. Wares *et al.* 2002; Chapman *et al.* 2007, 2008; Cunningham 2008)]. Although, our molecular evidence cannot precisely pinpoint whether *L. littorea* arrived with very early (i.e. Vikings) or later (i.e. Pilgrims) Europeans, it is apparent that these divergence estimates are consistent with the time frame and mechanism for human-mediated transport from Europe within the last several centuries.

While glaciation could also result in genetic bottleneck signatures in North America, our evidence argues against a preglacial existence for *L. littorea* in North America. First, our 1M divergence estimates between European and North American populations are many thousands of years later than the last glacial maxima (~20 000 years ago). Second, patterns for expansion following glacial refugia typically show low genetic diversity in the latitudes farthest from the source of the population expansion (Marko 2004). Our phylogenetic analysis of North American populations, which treated maritime Canada as a possible glacial refugial region (proposed as an alternative hypothesis to a recent introduction for the snail) compared against US populations found no difference in the amount of genetic diversity at either the regional level or at the site level, even when we compared Canadian sites vs. just the southernmost US sites. This pattern cannot simply be explained as the result of an under-sampling issue in Canada (as

we have shown was an issue we accounted for in Europe) because Chao2 haplotype estimates predict only a handful more haplotypes (~12) in Canada compared to the USA. In contrast to this result for *L. littorea*, a Pacific North American marine snail, *Nucella lamellosa*, showed evidence for a northern latitude glacial refugia based on an AMOVA test, which revealed a significant amount of subdivision between northern and southern latitudes in the snail (Marko 2004). Using the same technique, we did not observe significant  $F_{ST}$  or  $F_{CT}$  values between the Canadian and US subpopulations ( $F_{ST}$ : 0.0145;  $P = 0.20$ ;  $F_{CT}$ : -0.00038;  $P = 0.37$ ), suggesting little subdivision between northern and southern latitudes for North American *L. littorea*. This lack of subdivision not only argues against a glacial refugia, but it also suggests that the genetic similarity between the US and Canadian populations could be due to *L. littorea*'s pelagic larval dispersal, or it may suggest multiple introductions of the snail — or a combination of both hypotheses. Furthermore, the suggestion that *L. littorea* existed in glacial refugia until changes in the environment allowed the snail to expand its range in the 1800s (Wares *et al.* 2002) is supported neither by historical evidence nor by the experience of other species exhibiting similar range expansions (Chapman *et al.* 2007). Moreover, this scenario would require that the most conspicuous, dominant intertidal snail lay essentially quiescent for at least 10 000 years. Finally, other marine rocky intertidal species, *Semibalanus balanoides* (acorn barnacle) and *Mytilus edulis* (blue mussel), with similar larval dispersing mechanisms to *L. littorea* and believed to have existed in refugia during the last glaciation (Wares & Cunningham 2001), were not confined to maritime Canada following glacial retreat (as was the suggestion for a native North American origin for *L. littorea*). Taken together, these results argue strongly against a glacial refugia theory in maritime Canada for North American *L. littorea*.

Therefore, the most parsimonious conclusion of our results is a recent introduction of *L. littorea* to North America from Europe — this conclusion is not only based upon molecular patterns consistent with a recent founder event in the snail but also due to the convergent patterns we found in *L. littorea*'s associated trematode, *C. lingua*. *L. littorea*'s recent introduction was likely human-mediated due to its close association with human means of transport (e.g. through rock ballast; Carlton 1982), which may have been the mechanism of introduction to the northwest Atlantic for other intertidal species in the 19th century, like *Carcinus maenas* (Roman & Darling 2007) and *Fucus serratus* (Coyer *et al.* 2006), or *L. littorea* could have been intentionally introduced as a food source (Packard 1870; Carlton 1982). Furthermore, the snail's absence from North Atlantic islands, such as Iceland and Greenland, which are believed to have aided in the natural, stepping-stone invasions of several marine intertidal species following the last glaciation

(Johannesson 1988; Ingolfsson 1992) is further evidence that the snail did not move naturally across the North Atlantic. Additionally, our molecular genetic data are consistent with recent ecological patterns in trematode parasite species richness for *L. littorea* (Blakeslee & Byers 2008), which was significantly reduced in North America vs. Europe for *L. littorea* but not for native congeners, *Littorina saxatilis* and *Littorina obtusata*. In this investigation, the trematode species richness displayed in *L. littorea* was consistent with the well-established theory of enemy escape, suggesting a recent invasion from Europe for *L. littorea*, while congeners *L. saxatilis* and *L. obtusata* showed patterns that were instead consistent with an older, natural (likely stepping-stone) invasion from Europe across the North Atlantic into northeast North America.

More generally, our results further highlight the value of parasites to help resolve cryptogenic histories. Our complimentary parasite data set not only demonstrates that the trematode was likely introduced with its snail host, but it was also particularly important for resolving *L. littorea*'s cryptogenic history. This is because without our convergent parasite genetic information, *L. littorea*'s status as native or non-native may have continued to remain equivocal. For example even a study using multiple loci (Wares & Blakeslee 2007) was unable to effectively provide clear evidence for or against a recent introduction (although in this AFLP study, no fixed differences were detected between the two populations, adding some support to a recent invasion for the snail). Overall, our corroborative parasite analysis provided conclusive, convergent data, showing that *C. lingua* possessed the same level of diversity reduction and short divergence estimates as its snail host.

Altogether, our study highlights how genetics, and specifically in the case of our study, parasite genetics, can aid in the resolution of questionable invasion histories. Without the convergent genetic information we provided, *L. littorea*'s cryptogenic status could continue to be debatable. Similarly, other studies have used molecular tools to discern invasion histories when natural history and ecological information have not been sufficient to fully understand a species' presence in a region. For example, global cryptic invasions of the European green crab, *Carcinus maenas*, were distinguished using mitochondrial DNA (Geller *et al.* 1997). Additionally, DNA sequencing tools were used to resolve the questionable invasion history of the nassariid gastropod, *Cyclope neritea*, in the northwest Iberian Peninsula (Couceiro *et al.* 2008). Thus, molecular techniques have been shown to be powerful tools for understanding and elucidating questionable invasion histories — and specifically, our investigation of *L. littorea* was not only able to resolve a specific cryptogenic history, but it also demonstrates the success of the approach we applied here for resolving cryptogenic histories even for those introductions occurring hundreds of years ago.

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