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PII: S1567-1348(15)00283-X
DOI: http://dx.doi.org/10.1016/j.meegid.2015.07.021
Reference: MEEGID 2415

To appear in: *Infection, Genetics and Evolution*

Received Date: 7 April 2015
Revised Date: 1 July 2015
Accepted Date: 17 July 2015

Please cite this article as: Lawton, S.P., Lim, R.M., Dukes, J.P., Kett, S.M., Cook, R.T., Walker, A.J., Kirk, R.S., Unravelling the riddle of *Radix*: DNA barcoding for species identification of freshwater snail intermediate hosts of zoonotic digeneans and estimating their inter-population evolutionary relationships, *Infection, Genetics and Evolution* (2015), doi: http://dx.doi.org/10.1016/j.meegid.2015.07.021

Unravelling the riddle of *Radix*: DNA barcoding for species identification of freshwater snail intermediate hosts of zoonotic digeneans and estimating their inter-population evolutionary relationships

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Abstract

Radix spp. are intermediate host snails for digenean parasites of medical and veterinary importance. Within this genus, species differentiation using shell and internal organ morphology can result in erroneous species identification, causing problems when trying to understand the population biology of *Radix*. In the present study, DNA barcoding, using cox1 and ITS2 sequences, identified populations of *Radix auricularia* and *R. balthica* from specimens originally morphologically identified as *R. peregra* from the UK. Assessment of cox1 and ITS2 as species identification markers showed that, although both markers differentiated species, cox1 possessed greater molecular diversity and higher phylogenetic resolution. Cox1 also proved useful for gaining insights into the evolutionary relationships of *Radix* species populations. Phylogenetic analysis and haplotype networks of cox1 indicated that *Radix auricularia* appeared to have invaded the UK several times; some haplotypes forming a distinct UK specific clade, whilst others are more akin to those found on mainland Europe. This was in contrast to relationships between *R. balthica* populations, which had low molecular diversity and no distinct UK specific haplotypes, suggesting recent and multiple invasions from mainland Europe. Molecular techniques therefore appear to be crucial for distinguishing *Radix* spp., particularly using cox1. This barcoding marker also enables the population biology of *Radix* spp. to be explored, and is invaluable for monitoring the epidemiology of fluke diseases especially in the light of emerging diseases and food security.

Key words: *Radix auricularia*, *Radix balthica*, cox1, DNA barcoding, epidemiology, intermediate hosts, molecular diversity, phylogenetics

Abbreviations

π: nucleotide diversity; cox1: cytochrome oxidase 1; G: Gamma distribution; *h*: haplotype number; *Hd*: haplotype diversity; HKY: Hasegawa-Kishino-Yano nucleotide substitution model; I: invariable sites; ITS2: ribosomal internal transcribed spacer region 2; *K*: number of observed pairwise differences; ML: maximum likelihood; MP: maximum parsimony; NJ: neighbour joining; PCR: polymerase chain reactions; P-value: probability value; *p*-distance: pairwise distance; *S*: segregating sites; T93: Tamura Nei nucleotide substitution model; TS: transitions; TV: transversion

1. Introduction

Freshwater snails of the genus *Radix* are of considerable medical and veterinary importance as vectors of digenean parasites. *Radix* spp. are known to be intermediate hosts for schistosomatid blood flukes including avian parasites from the genus *Trichobilharzia* (Huňova et al., 2012) and the cattle parasite *Schistosoma turkestanicum*, which are agents of human cercarial dermatitis in Eastern Europe and Asia (Majoros et al., 2010). *Radix* also transmits the cosmopolitan re-emerging zoonotic disease echinostomiasis caused by echinostomatid flukes in South East Asia, significantly contributing to the global burden of intestinal trematodiasis (Georgieva et al., 2013; Saijuntha et al., 2011). However, perhaps the most important role for *Radix* in Europe and the UK is as intermediate hosts of *Fasciola hepatica* and *Fasciola gigantica*, agents of fascioliasis, causing reduced meat and milk production in cows, as well as morbidity in humans with more than 20 million human cases worldwide (Correa et al., 2011; Mas-Coma et al., 2009).

Accurate identification of such intermediate snail hosts is a prerequisite for understanding disease epidemiology and aids in focusing control, especially if resources are limited and parasites have a wide host range (Kane et al., 2008; Rollinson et al., 2009). Historically, species of *Radix* have been identified using shell morphology, reproductive system anatomy and colouration and these parameters continue to be used to describe new species (Glöer and Pešic, 2008). However, the utility of these morphological characters has been questioned due to the plasticity of shell morphology in response to environmental pressures and ontogeny (Huňova et al., 2012; Pfenninger et al., 2006). Recently, molecular taxonomic and phylogenetic approaches have been employed to resolve the relationships between *Radix* species and provide accurate identification (Bargues et al., 2001; Correa et al., 2011; Huňova et al., 2012; Pfenninger et al., 2006; Schniebs et al., 2011). Molecular-based taxonomy of disease vectors often contradicts traditional views and can result in extensive taxonomic revisions. For example several medically important snail species within the genus *Bulinus* were originally named and identified based on morphological characteristics, such as shell structure and internal anatomy, but have since been separated into distinct species based on molecular evidence (Kane et al., 2008). In fact, molecular approaches have aided in the elucidation of cryptic species and species complexes that appear morphologically identical (Pfenninger et al., 2006). Bargues et al. (2001) highlighted that eight species of *Radix* of medical/veterinary concern were thought to occur in central and northern Europe including *R.*

auricularia, R. ampla, R. balthica, *R. lagotis*, *R. labiata*, *R. ovata,* and *R. peregra.* However, molecular analysis of ribosomal DNA sequences indicated that *R. peregra*, *R. ovata* and *R. balthica* were the same species (Bargues et al., 2001). This reclassification is still debated in the literature with some researchers continuing to use the three separate names (Huňova et al., 2012; Pfenninger et al., 2006).

In an attempt to resolve taxonomic issues and to improve the accuracy of identification of *Radix* and other lymnaeids, several molecular markers have been used primarily based on the their ease to amplify and sequence, as well as the number of comparable published sequences available in public databases (Casiraghi et al., 2010). Such markers have included the nuclear ribosomal gene 18s, and the internal transcribed spacer regions ITS1 and ITS2 (Bargues et al., 2001) as well as the international barcoding gene cytochrome oxidase 1 (cox1) and ribosomal 16s (Correa et al., 2010; Pfenninger et al., 2011). Historically, both ITS regions have been used to differentiate between species of aquatic snail intermediate hosts of schistosomes that cause human urinary and hepatointestinal African schistosomiasis; including *Bulinus* and *Biomphalaria*, respectively (Dejong et al., 2003; Kane et al., 2008; Raahauge and Kristensen, 2000). However, Kane et al. (2008) and Dejong et al. (2003) argued that mitochondrial genes such as cox1 and 16s were more effective at differentiating species than nuclear genes, and could provide a biogeographical perspective because of their higher rate of evolution. Cox1 has been used to differentiate between species and populations of snails including *Indoplanorbis exustus* (Liu et al., 2010), *Theba pisana pisana* (Däumer et al., 2012) and species of *Pomacea* (Hayes et al., 2008; Lv et al., 2013). However, only a few studies have attempted to disentangle the relationship within and between species of *Radix* using mitochondrial markers. Pfenninger et al. (2006) defined operational taxonomic units using cox1, and later investigated the phylogeography and population structure of *R. balthica* in Northern Europe (Pfenninger et al., 2011).

With the exception of *R. balthica* (Pfenninger et al., 2011), there are no recent studies on the population biology, taxonomy and origins of *Radix* species in the UK, nor are there standardised techniques to identify and distinguish between morphologically similar species. This study aimed t employ DNA barcoding approaches to accurately identify morphologically plastic species of *Radix* and to compare and contrast the utility of cox1 and ITS2 sequences as markers for species identification. Additionally, cox1 sequences were used to provide insights

into the evolutionary relationships between populations of *Radix* species from the UK and Eurasia in order to test the utility of cox1 in providing phylogeographical inferences.

2. Materials and Methods

2.1 Collection and digenean screening of snails from UK waters

Freshwater snails were collected between June–September from Tundry Pond, Hampshire in 2011 and 2012 (n = 391), Pensthorpe Park, Norfolk in 2011 (n = 200), lochs in Wester Ross, Scotland in 2011 (n = 12), Ham Dip Pond (n =6), and Pen Ponds in Richmond Park , Surrey (n = 53) in 2012 during digenean parasite surveys. Snails were collected using scoop nets or by hand and were identified in the field as being either *Lymnaea stagnalis* or *Radix peregra* based on shell morphology. They were then taken to the laboratory and screened for cercarial infection as described in Lawton *et al*. (2014). Those individuals positive for digenean infection were killed by freezing at -20°C and preserved in 70–100% ethanol. Those snails not emitting parasites were screened every day for two weeks to check for latent infection.

2.2 DNA extraction, PCR amplification and assembly of cox1 and ITS2 fragments

A total of 21 snails, preliminarily identified as *R. peregra*, were infected with digeneans; small tissue snips were taken from the head-foot of these snails for DNA extraction. Snail tissue was homogenised in ATL buffer (Qiagen Inc.) and DNA was subsequently extracted using the DNeasy tissue kit (Qiagen Inc.) following the manufacturer's recommendations with an extended initial digest of 24 h. For each specimen, a partial fragment of the mitochondrial cox1 gene was amplified with PCR using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA) and protocols described by Folmer et al. (1994) and the ITS2 region was amplified using primers NEWS (5'-TGTGTCGATGAAGAACGCAG-3') and RIXO (5'-TTCTATGCTTAAATTCAGGGG-3') and protocols of Almeyda-Artigas et al. (2000). PCR reactions were performed with each primer set independently using 2 µl 10µM of each primer, 12.5 µl of DreamTaq^{TM} PCR master mix (2X DreamTaq buffer, 0.4 mM of each dNTP, 4mM $MgCl_2$) and 1-2 ng/µl DNA, with final reactions made up to 25 µl with PCR grade water. Reactions were performed using a Veriti 96 well thermal cycler (Applied Biosystems^{TM}). Each amplicon was visualized on a

1% agarose gel stained with GelRed (Bioline) using 5 µl of PCR product. The remaining 20 µl PCR products were sequenced at the Natural History Museum, London, using fluorescent dye terminator sequencing kits (Applied Biosystems™), then run on an Applied Biosystems 3730XL automated sequencer.

2.3 Molecular identification of species and phylogenetic reconstruction

DNA sequences of cox1 (Accession: XXXX – XXXX) and ITS2 (Accession: XXXX – XXXX) were assembled and edited using Bioedit (Hall, 1999) and BLAST searches performed at NCBI (http://www.ncbi.nlm.nih.gov/) against the GenBank sequence database for initial identification of snails and to ensure no contamination from other organisms and initial BLAST searches identified sequences generated in this study to be most similar to *R. auricularia*, *R. balthica* or *R. peregra*. For detailed phylogenetic identification of species generated sequences were aligned with taxonomic representatives of published sequences of all available *Radix* definitive species using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Where possible an equal number of reference DNA sequences of cox1 and ITS2 for *Radix ampla*, *Radix auricularia*, *Radix balthica*, *Radix labiata*, *Radix natalensis* and *Radix rubignosa* were used. This ranged from a single sequence to 14 representative sequences depending on availability and it is important to note that it was not always possible to obtain both cox1 and ITS2 sequences from the same individual from the same locality for most species as this information was not available in the GenBank database. Although an exhaustive search of *Radix* sequences was performed, due to the disproportionate number of sequences available for these snail species, with only 2 cox1 sequences were available for *R. peregra* and *R. natalensis* compared to over 800 for *R. balthica* for example. A maximum limit of 14 reference sequences was used as this was in excess of reference sequences used in other molecular identification studies of *Radix* species (Bolotov et al., 2014; Dung et al., 2013; Jouet et al., 2008, 2010; Schniebs et al., 2011, 2013) and other snails of medical and veterinary importance (Correa et al., 2011; Kane et al., 2008; Kulsantiwong et al., 2013). This number of reference sequences would be sufficient for distinct species specific clades to emerge during phylogenetic analysis and definitively identify sequences generated in this current study. Each of these reference sequences was considered as putative throughout the analysis and were chosen because they were generated for studies focused primarily on the molecular identification and the evolutionary relationships of *Radix* species particularly from the following works: Albrecht et al., 2009;

Bargues et al., 2001; Bolotov et al., Christiansen et al., 2014; Cipriani et al., 2011; Correa et al., 2011; Dung et al., 2013; 2014; Ferreira et al., 2014; Ferté et al., 2005; Huňova et al., 2012; Jouet et al., 2008, 2010; Kaset et al., 2009; Liu et al., 2010; Patel et al., 2015; Pfenninger et al., 2011; Novobilsky et al., 2014; Schniebs et al., 2011, 2013; Vinarski et al., 2015; von Oheimb et al., 2011;

Therefore the sequences used for inter-species comparisons were considered to be representative of taxa alone and were not used to make biogeographical inferences. Phylogenetic analysis was performed in MEGA 5 (Tamura et al., 2011) and *Lymnaea stagnalis* was used as an outgroup. Character based phylogenetic analysis was performed including both maximum parsimony (MP) and maximum likelihood (ML) methods. For both markers MP phylogenies were constructed from a consensus tree inferred from 10000 most parsimonious trees obtained using the close–neighbour-interchange algorithm with a search level of 3 in which initial trees were obtained with random addition of sequences over 3000 replicates. For ML analysis, evolutionary substitution models were identified for each marker in MEGA 5 with cox1 evolving under the conditions of the HKY+G model and ITS2 evolving under the conditions of the T93+I model. These models had the lowest Bayesian information criterion relative to other models and were then used to reconstruct ML phylogenies. In all phylogenetic analyses described, bootstraps were calculated from 1000 replicates during tree construction to provide nodal support values.

2.4 Assessment of marker suitability and inter- and intra-species molecular diversity

To compare the molecular characteristics of cox1 and ITS2 between species and assess their utility as markers for species identification, substitutional changes and molecular diversity were measured. Molecular diversity between species was determined using DnaSP 5 (Librado and Rozas, 2009). General measures of nucleotide diversity were calculated including number of segregating sites (S), nucleotide diversity (π), and average pair wise comparisons (K). The uncorrected *p*-distance was calculated between each species and sequence using MEGA 5 and the frequency of transition (TS) and transversion (TV) mutation within each comparison determined. Sequence substitution saturation was also calculated using the entropy-based index implemented in the program DAMBE (Xia et al., 2003) enabling TS and TV values to be plotted against *p*-distance for each marker. Occurrence/identification of evolutionary

"noise" could then be identified in the form of substitutional saturation, which could have affected phylogenetic reconstruction and accurate identification of species.

To provide insights into the evolutionary relationships between populations of *Radix* spp. collected in this study and other geographical isolates from Eurasia, the cox1 sequences were compared to previously published sequences of the same species from different localities. The majority of available phylogeographical data for snails are based on cox1 sequences; several authors have indicated the excellent ability of the marker to differentiate geographical lineages in other species (Hayes et al., 2008; Kane et al., 2008; Lv et al., 2013). Cox1 sequences were aligned using the same method as above with published sequences representing different geographical regions within and between countries where possible Molecular diversity within the alignment was calculated as above and the number of haplotypes (h) and haplotype diversity (Hd) were considered. Phylogenetic analysis was initially performed using the MP approach as previously described using MEGA alongside the generation of parsimonious haplotype network gene genealogies in TC1.21 (Clement et al., 2008), as reported in Schniebs et al. (2011; 2013), to assess the evolutionary relationships between snails from different geographical locations.

3. Results

3.1 Phylogenetic species identification based on cox1 and ITS

The cox1 sequences produced an alignment of ~570 bp without gaps. In contrast, the ITS2 sequences produced an alignment of 439 bp after editing as a result of length variation within and between species. Despite these differences, maximum likelihood phylogenetic analysis of cox1 and ITS2 (Fig. 1 and 2) provided the same overall inter-species relationships with two major groups occurring. The "*R. balthica"* group contained a mixed clade of *R. balthica* and *R. peregra*, with a sister clade made up of all reference sequences of *R. ampla*. *Radix labiata* also fell into the "*R. balthica*" group appearing as a distinct sister taxa to the *R. balthica*/*R. peregra* + *R. ampla* subclade. The "*R. auricularia"* group contained *R. auricularia*, *R. natalensis* and *R. rubiginosa.* Within the "*R. auricularia"* group the cox1 analysis showed that *R. natalensis* forms a distinct sister group to a *R. rubiginosa* + *R. auricularia* subclade which is contrasted in the ITS2 analysis with *R. natalensis* and *R. rubiginosa* forming a subclade which appears as a sister group to *R. auricularia* (Fig. 1 and 2). In the maximum

parsimony analysis the ITS2 alignment provided an overall tree topology which was identical to the ML phylogeny, but with greater nodal support of bootstrap >50. This was not the case for the cox1 marker. Despite high nodal support, the MP phylogenies indicated that *R. natalensis* and *R. rubiginosa* were more closely associated with the *R. balthica* group. The general topological patterns in the trees did agree with other published phylogenies (e.g. Correa et al., 2010; Remigio, 2002; Schniebs et al., 2011, 2013). Also, the cox1 phylogenies showed greater nodal support with 20 nodes in the ML analysis and 18 nodes in the MP analysis being supported with bootstraps of >50 compared to 14 nodes in the ML and 16 MP phylogenetic analyses of ITS2. The cox1 tree shows a greater level of topological structuring with well supported sub-clades of *R. balthica/R.peregra* and *R. auricularia* forming which are absent in the ITS2 phylogeny (Fig. 1 and 2). However, regardless of these suitable differences in tree topologies, both the cox1 and ITS2 sequences generated from snails in this study clearly separate between the two species and can be identified as *R. balthica* and *R. auricularia* with SnUK1 – 6 and SnUK 15 – 16 clustering with all reference sequences of *R. auricularia* and SnUK 7 – 14 and SnUK 17 – 21 with all reference sequences of *R. balthica/R. peregra*.

The current phylogenetic analysis has not differentiated *R. balthica* and *R. peregra* as distinct species (Fig. 1 and 2). A lack of genetic differentiation was also illustrated between the *p*-distances of unique cox1 and ITS2 haplotypes within the *R. balthica* clade (Table 1) with an average divergence between *R. balthica* and *R. peregra* cox1 sequences of 1.21 % and 0.2 % for ITS2 sequences (Table 1). Detailed cox1 analysis revealed 19 unique haplotypes within the *R. balthica/R.peregra* clade which were represented by a single cox1 sequence of *R. peregra* (JN614401), 18 *R. balthica* sequences, of which 12 were from published works and 6 were generated in this study (SnUK 11, 17 – 21). When the only *R. peregra* specific haplotype was compared to all other *R. balthica* haplotypes within the clade, again only a low level of divergence was recorded ranging from 0.5 – 2%. Of the 34 ITS2 sequences which make up the majority of the *R. balthica/R.peregra* clade, only six unique haplotypes were identified represented by 2 *R. balthica* haplotypes (HE573086, HQ003232), 2 *R. peregra* haplotypes (AJ319634, KF887039) and 2 novel sequences generated in this study (samples SnUK 9 and SnUK 18) (Table 3). Across the unique ITS haplotypes the divergence was substantially lower than that seen in the cox1 ranging from 0.5 – 0.9% (Table 3). Therefore, all sequences within the *R. balthica/R. peregra* group will be considered as *R. balthica* based on the current cox1 and ITS2 analysis

3.2 Molecular diversity and characteristics of cox1 and ITS2 alignments

When cox1 and ITS2 were compared, nucleotide diversity and divergence were consistently higher in cox1 with marked differences seen in the number of segregating sites, nucleotide diversity, pairwise divergence and specific substitutions (Table 4a). There appeared to be no substitutional saturation detected in the cox1 alignment (Iss: 0.318< Iss.c: 0.740, P = 0.000), nor the ITS2 alignment (Iss: 0.0725 < Iss.c: 0. 71225, P = 0.000) (Table 4b). When TV and TS values were plotted against *p*-distance, again there was no major indication of saturation of a particular substitutional type for cox1 or ITS2 (Fig. 3). Importantly, cox1 alignment consistently showed a greater number of substitutions than ITS2 as *p*-distance increased (Fig. 3). Overall, the lack of saturation, and/or differences in the number of substitutions, indicate that all substitutional types had phylogenetic value and that both markers are not only suitable for species identification, but also for accurate phylogenetic reconstruction (Table 4b).

3.3 Evolutionary relationships between geographical isolates of *Radix auricularia* and *Radix balthica*

The cox1 alignments consistently displayed greater nucleotide diversity and divergence across eight *R. auricularia* from Hampshire and Surrey (Pen Ponds) and thirteen *R. balthica* from Scotland, Norfolk and Surrey (Ham Dip Pond). Although more *R. balthica* were analysed, *R. auricularia* displayed greater diversity for both markers (Table 5). Due to the high level of variation in the cox1 and a lack of corresponding ITS2 and Cox1 sequences from previous studies, cox1 sequences were used for geographical comparisons of haplotype diversity. Thirty three cox1 sequences of *R. auricularia* were compared between ten countries ranging across Eurasia. The eight UK sequences were represented only by data in this study as no previously published sequences were found. Twenty three individual haplotypes were identified and a high level of molecular diversity (S = 88; π = 0.03289) and divergence (K = 14.07765) were apparent (Table 5). Fifty five sequences of *R. balthica* were compared between five countries; the majority of the sequences (21 sequences + 13 sequences from this study) were from the UK. Thirty four novel haplotypes were identified and relatively low levels of molecular diversity (S = 105; π = 0.01487) and divergence (K= 7.51044) were seen (Table 5).

In order to identify relationships between different geographical isolates, phylogenetic analysis was performed using the cox1 sequences of *R. auricularia* and *R. balthica*, due to the considerably higher diversity of cox1 than ITS2. The *R. auricularia* phylogeny separated into three major clades with clade 1 containing all sequences from mainland Europe and the UK (Fig. 4). A second major clade (clade 2) consisted of individuals from Russia and Tajikistan and a final third clade (clade 3) was composed of a single individual sequence from Armenia (JN794353). Within clade 1 the UK snails separated into two subclades (SC), a European clade (SC1) made up of haplotypes from Croatia, Montenegro, Albania, Russia, Greece, France, Spain, Armenia and SnUK 1 - 2 from Hampshire, UK. A second UK specific subclade (SC2) contained all the other UK haplotypes (Fig. 4a). The same interrelationships between *R. auricularia* haplotypes were also seen in haplotype network analysis and again the UK haplotypes fell into the two distinct clades with a UK specific grouping and a single haplotype falling into a European group (Fig 4b). However, the haplotype network analysis showed the Russian and Tajikistan sequences to form a distinct group from the European sequences with the exception of Russian haplotype JN794508 which showed to be more closely akin to haplotypes from Albania (EU818819) and Greece (EU818811) in both the phylogenetic and network analysis (Fig. 4a and b). Interestingly, JN794353 from Armenia, which formed a unique clade in the MP phylogeny, appeared to cluster with the other European haplotypes in the network analysis but did form a unique discrete lineage.

Unlike *R. auricularia* no discrete geographical specific clades were observed in the MP phylogeny of the *R. balthica* haplotypes (Fig. 5a). Only a single haplotype was seen from Norfolk which was also location specific; the two haplotypes from Wester Ross were also geographically specific. However, one of the haplotypes, H11a, from Surrey was shared between snails from Buxton in the North West of England and also from Sweden (Fig. 5a). With the exception of HQ244929 which formed a distinct lineage, the lack of divergence and distinct clades in both the phylogenetic and haplotype network analysis illustrates the close relationship between sequences from mainland Europe and the UK (Fig. 4a and 4b).

4. Discussion

4.1 Molecular phylogenetics for identifying *Radix* species

The molecular characterisation of *Radix* species contradicted the original identifications based on shell morphology. Phylogenetic analysis of both cox1 and ITS2 sequences identified snails as *R. balthica* and *R. auricularia* which differentiated into two distinct clades, the *R.balthica* group a major clade containing only species from Europe, and the *R. auricularia group* containing African and Eurasian species. This provides further evidence to suggest that *R. balthica* is a Palaearctic species distributed throughout Europe from Iceland to Mediterranean countries (Schniebs et al., 2011). The clustering of *R. auricularia* with the African *R. natalensis* and the Asian *R. rubigonosa* was interesting indicating a potential Old World origin. Although it is not possible to speculate an Asian or European origin of *R. auricularia*, the snail does show a greater distribution than *R. balthica* being found throughout Eurasia and is common in Europe and the near East, parts of South East Asia including Thailand and the Indian subcontinent (von Oheimb et al., 2011). Only extensive phylogenetic analysis with multiple nuclear and mitochondrial markers will elucidate true species inter-relationships within the genus and resolve many of the remaining taxonomic issues (Bargues et al., 2001; Correa et al., 2011; Pfenninger et al., 2006).

Interestingly, *R. labiata* formed a distinct well supported clade within the *R. balthic* group separating it from *R. balthica* and *R. peregra*. Schniebs et al. (2013) had highlighted that *R. labiata* had been synonymised with *R. peregra* and also had close taxonomic affinity with *R. balthica*. *Radix labiata* does share an over-lapping distribution throughout Europe with *R. balthica* and *R. peregra* and the synonomysing of *R. labiata* and *R. peregra* was initially on based on overlapping morphological characters of shell and internal anatomy (Schniebs et al., 2013). However, the phylogenetic separation between *R. labiata* and *R. peregra* indicate that they are separate species. This is also supported by high levels of divergence in the cox1 with *R. labiata* and *R. peregra* at 12.12% and also between *R. labiata* and *R. balthica* 10.33%. This is considerably higher than the typical 5% divergence in mitochondrial genes between mollusc species as illustrated in studies on *Eobania* species (Desouky and Busais, 2012), *Goniobasis* species (Dillon and Frankis, 2004), *Helixena* species (Van Riel et al., 2005), *Iberus* species (Elejalde et al., 2008), and *Rhagada* species (Johnson et al., 2013) However, reference sequences of *R. balthica* and *R. peregra* clustered together in both cox1 and ITS2 phylogenies showing no differentiation between species, which was also confirmed with a low cox1 divergence of 1.21%, far below the typical 5% divergence between species. This is consistent with other studies where *R. balthica*, *R. peregra* and *R. ovata* could not be differentiated based on molecular data (Bargues et al., 2001; Huňova et al., 2012).

Wullschleger and Jokela (2002) suggested that *R. peregra* and *R. ovata* were different from *R. balthica* due to their different growth rates and reproductive schedules in similar environments and avoidance of crossing with apparent 'other' species from sympatric populations. However, partitioning of snail populations is well known for some species showing high morphological and reproductive heterogeneity with several phenotypes being sympatric, but indistinguishable as species at the molecular level including the land snail *Cepea nemoralis* (Cook, 1998; Ochman *et al.,* 1983) and the fresh water snails within the *Bulinus* genus (Kane et al., 2008; Rollinson et al., 2009). *Radix balthica* shows adaptive responses in morphological and life history traits as a direct response to environmental factors (Pfenninger et al., 2006; Rundle et al., 2011; Schniebs et al., 2011) showing great divergence within species, but also considerable phenotypic overlap between others. This was particularly true for *R. balthica* and *R. auricularia,* illustrating that even within sympatric populations of distantly related species, phenotype convergence would occur as a result of similar selective pressures confounding characters used for taxonomic identification using traditional methods (Schniebs et al., 2011). This issue has also been highlighted in other freshwater snails including the *Bulinus forskalii* group whereby sympatric species are morphologically indistinguishable and can only be identified through molecular methods (Jones et al., 1997, 2001). Consequently, molecular barcoding is now standard practice for *Bulinus* species identification (Kane et al., 2008; Rollinson et al., 2009). Based on the results in this current study, *R. labiata* is a distinct species from *R. peregra*, and *R. balthica* and *R. peregra* are the same species. This also illustrates that reference sequences of *R. peregra* that are available on GenBank have clearly been produced for snails which have been mis-identified based on shell and/or internal anatomy.

4.2 Molecular markers for species identification and a role of DNA barcoding

Although several authors now agree that molecular taxonomy is essential for delineation and identification of *Radix* species (Correa et al., 2011; Huňova et al., 2012; Schniebs et al., 2011), there is a lack of consensus on the correct marker to employ. Major studies have employed ITS2, but have provided few alternatives. Bargues et al. (2001) illustrated that phylogenetic topological changes can occur when comparing several genera of lymnaeids, and although genera specific clades tend to occur, the interrelatedness of these clades could be confounded by the kind of phylogenetic analysis employed. The difficulty with using ITS2 sequences for phylogenetic analysis is primarily caused by sequence length variation between

species and low levels of variation between closely related species. Low differentiation between *R. balthica*, *R. lagotis, R. labiata* and *R. ampla* has been recorded (Schniebs et al., 2011) and confirmed in this study. In the current study, ITS2 differentiated between species and produced well-supported phylogenies. The cox1 tree, however, had greater nodal support producing phylogenies with longer branch lengths and each clade possessed several sub-clades within the *R. auricularia* and *R. balthica* data. Cox1 also showed greater molecular diversity and divergence than ITS2 in cross-species analysis and results tended to be concurrent with other studies that have used different mitochondrial markers for *Radix* identification and phylogenetics such as cyt-b (Schniebs et al., 2011, 2013) and 16s (Correa et al., 2010; Remigio, 2002). Cox1, therefore, appears to have a higher phylogenetic signal and can differentiate between closely related species more effectively than ITS2 (Pfenninger et al., 2006; Schniebs et al., 2011). This characteristic of mitochondrial markers has been seen consistently across the Planorboidea (Albrecht et al., 2007; Kane et al., 2008) with authors indicating a preference for cox1 and other mitochondrial markers. However, the higher rate of evolution of mitochondrial genes can cause problems when identifying taxa to species level. This is because of the risk of substitutional saturation affecting phylogenetic signal that can produce erroneous relationships and incongruence between DNA loci (Jørgensen et al., 2007). Here, no such saturation was identified in cox1 or ITS2 sequence data demonstrating that both markers are useful for phylogenetic reconstruction and species identification. Although both cox1 and ITS2 markers are as easy to amplify as each other from snail material based on the differences in nodal support and uncorrected p-distance estimates of divergence, cox1 should preferentially be used over ITS2.

4.3 Evolutionary inter-relationships between UK *Radix auricularia* and *R. balthica* and populations from mainland Europe

Although fewer *R. auricularia* were identified in the present study, higher molecular diversity existed within cox1 and ITS2 sequences relative to those of *R. balthica*. When *R. auricularia* sequences were compared with those from other localities, three distinct clades were formed with a European clade (clade 1) an Asiatic clade (clade 2) with snails from Russia and Tajikistan and a third clade (clade 3) containing only single sequence from Armenia (JN794353). These groups were also well supported in the haplotype network with each of the major clades reappearing along with same subclades. It is clear that there is a definite Eurasian distribution of *Radix auricularia* and the separation between clades 1 and 2 is likely

to be due to the effect of geographical divergence as the Russian sequences in SC4 of clade 2 were generated from populations on the far Eastern coast of Russia, in Kamchatka (Bolotov et al., 2014) and those in SC5 of clade 2 are from the Russia boarders of Kazakhstan (von Oheimb et al., 2011). Interestingly, JN794508 from Russia clusters into clade 1 more akin with haplotypes from Albania and Greece. The position of JN794508 further illustrates the importance of geographical separation on genetic differentiation in *R. auricularia* as this particular sample was taken from the Krasnodar, where Russia boarders the Black Sea (von Oheimb et al., 2011).

Both the phylogenetic analysis and haplotype network of *R. auricularia* indicate two distinct groups of the snails in the UK which could be indicative of multiple historical invasion events of *R. auricularia* into the UK. The presence of a UK specific subclade SC2 may have occurred as a result of long term isolation of populations from mainland Europe as a consequence of the separation of the UK from the European landmass at the end of the last ice age. Freshwater gastropod populations diverge at high evolutionary rates, not only because of rapid generation times, but also as a result of self-fertilisation causing excessive genetic drift as seen with the planorbids *Bulinus truncatus* (Djuikwo-Teukeng et al., 2011) and *Physa acuta* (Bousset et al., 2004). However, as stated previously, there is a UK haplotype more akin to those from mainland Europe. This haplotype, from Hampshire, may have invaded and established relatively recently. The Hampshire locality is a feeding site for migratory birds, particularly those from mainland Europe. Considerable evidence exists that birds traffic snail eggs and juveniles on feathers and feet (Figuerola et al., 2005); snails might also survive passage through bird digestive systems (Wada et al., 2012). Such translocation could account for some of the relations between UK specific populations of *R. auricularia* and also those of other populations within the European clade. This may explain disparities in relationships between haplotypes and geographical locations.

The molecular diversity of *R. balthica* was considerably lower than that of *R. auricularia*, most likely because the comparative data available was generated from a few populations across Europe and there was over-representation of *R. balthica* from the UK in the analysis. However, unlike *R. auricularia*, *R. balthica* appears to be restricted to Europe and populations are likely to be recovering from bottle-neck events caused during the last ice age (Pfenninger et al., 2006; 2011). Haplotypes and lineages appeared to be shared between the UK and European countries with few country specific clades emerging. There did not appear to be any

geographically distinct lineages when *R. balthica* cox1 sequences were compared with others suggesting recent rapid divergence between the mainland European and UK populations and between populations in the UK. Similar relationships between populations of *R. balthica* have also been described by Pfenninger et al. (2011) possibly as a result of passive dispersal by water birds. Such dispersal events would have resulted in multiple invasions from mainland Europe and continual movement of snails around the UK diluting effects of genetic drift and other population processes by the continual input of novel genotypes into previously colonised habitats (Pfenninger et al., 2011). Thus, UK *R. balthica* populations may have multiple origins and sufficient isolation/time has not yet to allow subpopulation structuring with reduced diversity, a result of most populations establishing recently, primarily through self-fertilisation.

The cox1 marker has provided excellent inter-population resolution and can deliver substantial phylogeographical signal to measure differentiation between geographical isolates. Although cox1 indicates that there could be have been several potential origins for both *Radix* species in the UK, based on the findings from this study, it could be suggested that *R. auricularia* populations are probably far older and more established than *R. balthica* populations due to increased phylogenetic resolution and structuring.

4.4 Conclusion and final remarks

Molecular identification is the only reliable method to identify *Radix* species as shell and other anatomical features are morphologically plastic and most *Radix* species share morphological characters as a result of convergent adaptations to shared environments. Therefore, without extensive expertise in malacology, accurately distinguishing relationships between species is impossible. However, if molecular approaches are to be embraced, a detailed understanding of the taxonomic and phylogenetic power of molecular markers and agreement on which specific markers should be used is needed. As with other freshwater snails, cox1 and ITS2 markers were excellent for differentiating species of *Radix*, but cox1 appears to be superior for phylogenetic species delineation. The cox1 marker is also useful for providing evolutionary insights into the population biology of *Radix* species allowing the measurement of genetic differentiation between snail populations and historical movements and colonisations. Molecular approaches to elucidate the taxonomy and population biology of medically and veterinary important snails is becoming a fundamental prerequisite to

understand the epidemiology of disease and is now enabling the focusing of limited control resources (Kane et al., 2008; Rollinson et al., 2009). Currently there is a considerable lack of knowledge of diversity of snail vectors of disease in the UK and, only through further focused studies of snail species and their populations, will a true understanding of the epidemiology and risk of fluke infection be achieved. This is of particular importance in the light of the increase in the prevalence of food borne zoonosis in the UK such as *Fasciola* (Gordon et al., 2013; Howell et al., 2015), those with potential impact on food security such as *Diplostomum* species which cause major problems for fish farms in Northern Europe (Hakalahti et al., 2006), and the emergence of unknown infections such as agents of swimmers itch in UK recreational waterways (Lawton et al., 2014). All of which could have long term economic impacts.

Competing interests

The authors have no competing interests to declare.

Authors' contribution

SPL, JPD, SMK, RTC, AJW, RSK were involved in conceiving the project and wrote the manuscript. SPL, RSK, JPD, AJW and RC performed the field collection, and SMK, RTC, RSK identified snails morphologically. SPL, RMO and JPD performed the molecular laboratory work and phylogenetic analysis. SPL and RMO performed the in-depth bioinformatics analysis and population level analysis.

Acknowledgements

The authors would like to thank Mrs J. Llewellyn–Hughes and her staff for performing the DNA sequencing reactions at the Molecular Sequencing Facility at the Natural History Museum, London. We would also like to thank Kingston University for partial funding of the molecular work through a summer intern studentship to RMO. We are grateful to Mr Revell, The Four Seasons Hotel, Hampshire, Pensthorpe Park, Norfolk and The Royal Parks for permission to sample snails.

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Figure legends:

Figure 1: Phylogenetic reconstruction of the genus *Radix* and identification of *Radix* species in the UK based on cox1. Species of *Radix* were determined based upon the phylogenetic clustering of DNA sequences. Phylogenetic reconstruction of species based on cox1 sequences shows the identification of UK *Radix* species separating into defined *Radix auricularia* and *R. balthica*. Phylogenetic trees presented are constructed a) using maximum likelihood (ML) character based methods constructed under the conditions of the HKY+G

model and b) MP phylogenies constructed from a consensus tree inferred from 10000 most parsimonious trees obtained using the close–neighbour-interchange algorithm with a search level of 3 in which initial trees were obtained with random addition of sequences over 3000 replicates. The nodal support values shown are the boot strap values from each analysis based on 1000 replicates with a 50% cut off.

Figure 2: Phylogenetic reconstruction of the genus *Radix* and identification of *Radix* species in the UK based on ITS2. Species of *Radix* were determined based upon the phylogenetic clustering of DNA sequences. Phylogenetic reconstruction of species based ITS2 sequences shows the identification of UK *Radix* species separating into defined *Radix auricularia* and *R. balthica*. Phylogenetic trees presented are constructed using maximum likelihood constructed under the conditions of the T93+I model and b) MP phylogenies constructed from a consensus tree inferred from 10000 most parsimonious trees obtained using the close–neighbour-interchange algorithm with a search level of 3 in which initial trees were obtained with random addition of sequences over 3000 replicates. The nodal support values shown are the boot strap values from each analysis based on 1000 replicates with a 50% cut off.

Figure 3: Nucleotide substitutional plots of transitions (TS) and transversions (TV) for *Radix* species and outgroup *Lymnaea stagnalis* relative to genetic distance (*p-distance*). TS/TV plot of a) cox1 and b) ITS2. There is an overlap of TS and TV in both cox1 and ITS2 indicating no substantial substitutional saturation within the markers when compared across species.

Figure 4: Phylogenetic comparisons of cox1 haplotypes from several geographical isolates of *Radix auricularia*. a) Maximum parsimony (MP) reconstruction of cox1 haplotypes of *Radix auricularia*. b) Most parsimonious haplotype network of cox1 haplotypes of *R. auricularia* where the size of a shaded circle represents the numbers of sequences within a specific haplotype.

Figure 5: Phylogenetic comparisons of cox1 haplotypes from several geographical isolates of *Radix balthica.* a) Maximum parsimony (MP) reconstruction of cox1 haplotypes of *Radix balthica*. b) Most parsimonious haplotype network of cox1 haplotypes of *R. balthica*

where the size of shaded circle represents the numbers of sequences within a specific haplotype. Both analyses illustrate the lack of defined genetic differentiation between localities in the UK and mainland Europe.

Tables:

Table 1: Average uncorrected p-distance of cox1 in the bottom left corner and ITS2 in the top right corner between *Radix* species used in the phylogenetic analysis

	Lymnaea stagnalis	*R. auricularia*	*R. rubiginosa*	*R. natalensis*	*R. labiata*	*R. ampla*	*R. balthica*	*R. peregra*
Lymnaea stagnalis		0.29632 (SD±0.00717)	0.30120 (SD±na)	0.29915 (SD±na)	0.27500 (SD±0.001113)	0.27105 (SD±0.001855)	0.29628 (SD±0.001441)	0.28541 (SD±0.001101)
R. auricularia	0.17153 (SD±0.003225)		0.064 (SD±0.010193)	0.063 (SD±0.011787)	0.109 (SD±0.018418)	0.117 (SD±0.020671)	0.125 (SD±0.01605)	0.122 (SD±0.016978)
R. rubiginosa	0.16637 (SD±na)	0.13305 (SD±0.00508)		0.028 (SD±na)	0.110 (SD±0.004945)	0.127 (SD±0.003638)	0.124 (SD±0.0043)	0.121 (SD±0.002721)
R. natalensis	0.16913 (SD±0.00124)	0.14236 (SD±0.01892)	0.138205 (SD±0.001105)		0.118 (SD±0.00628)	0.127 (SD±0.00310)	0.133 (SD±0.005624)	0.132 (SD±0.003985)
R. labiata	0.15221 (SD±0.00289)	0.13684 (SD±0.04712)	0.15548 (SD±0.004407)	0.13399 (SD±0.004622)		0.075 (SD± 0.005217)	0.074 (SD±0.017246)	0.076 (SD±0.010072)
R. ampla	0.13230 (SD±0.0011)	0.12782 (SD±0.03940)	0.14922 (SD±0.001474)	0.13480 (SD±0.00622)	0.09593 (SD±0.032076)		0.045 (SD±0.00951)	0.047 (SD±0.005008)
R. balthica	0.16247 (SD±0.003075)	0.13821 (SD±0.019317)	0.14695 (SD±0.002355)	0.13320 (SD±0.006245)	0.10330 (SD±0.0326)	0.08535 (SD±0.018989)		0.002 (SD±0.002555)
R. peregra	0.10625 (SD±0.09201)	0.14861 (SD±0.0042)	0.1521 (SD±na)	0.13996 (SD±0.007115)	0.12120 (SD±0.005628)	0.09283 (SD±0.0014)	0.01210 (SD±0.004054)	

Table 2: Uncorrected p – distances between sequences of unique cox1 haplotypes between *R. balthica* and *R. peregra* represented as p – distance in the bottom left and numbers of nucleotide substitutions between sequences in the top right

	JN614401 *R. peregra*	KP242666 *R balthica*	KP242465 *R. balthica*	KP242850 *R. balthica*	KP242782 *R. balthica*	KP242756 *R. balthica*	KP242646 *R. balthica*	KP242320 *R. balthica*	HQ244855 *R. balthica*	HQ244977 *R. balthica*	HQ244915 *R. balthica*	HQ244935 *R. balthica*	HQ244783 *R. balthica*	SnUK 11	SnUK 17	SnUK 18	SnUK19	SnUK 20	SnUK 21
JN614401 *R. peregra*		4	3	5	7	6	6	3	7	11	6	7	5	9	9	8	7	7	6
KP242666 *R. balthica*	0.007		3	6	5	4	5	3	5	9	4	5	5	7	7	6	5	5	4
KP242465 *R. balthica*	0.005	0.005		6	6	5	5	4	6	10	5	6	3	8	8	7	6	6	5
KP242850 *R. balthica*	0.009	0.01	0.01		5	4	3	5	5	9	4	5	4	7	7	6	5	5	4
KP242782 *R. balthica*	0.012	0.009	0.01	0.009		3	4	6	4	8	3	4	4	6	6	5	4	4	3
KP242756 *R. balthica*	0.01	0.007	0.009	0.007	0.005		3	5	3	7	2	3	3	5	5	4	3	3	2
KP242646 *R. balthica*	0.01	0.009	0.009	0.005	0.007	0.005		6	4	8	3	4	3	6	4	5	4	4	3
KP242320 *R. balthica*	0.005	0.005	0.007	0.009	0.01	0.009	0.01		6	10	5	6	6	8	5	7	6	6	5
HQ244855 *R. balthica*	0.013	0.009	0.011	0.009	0.007	0.006	0.007	0.011		6	3	2	4	3	7	2	1	4	3
HQ244977 *R. balthica*	0.02	0.017	0.019	0.017	0.015	0.013	0.015	0.019	0.011		7	6	7	7	2	6	5	8	7
HQ244915 *R. balthica*	0.011	0.007	0.009	0.007	0.006	0.004	0.006	0.009	0.006	0.013		3	3	4	6	3	2	3	2
HQ244935 *R. balthica*	0.013	0.009	0.011	0.009	0.007	0.006	0.007	0.011	0.004	0.011	0.006		4	3	3	2	1	4	3
HQ244783 *R. balthica*	0.01	0.01	0.006	0.008	0.008	0.006	0.006	0.012	0.008	0.013	0.006	0.008		5	5	4	3	4	3
SnUK 11	0.016	0.012	0.014	0.012	0.01	0.009	0.01	0.014	0.006	0.013	0.007	0.006	0.01		4	3	2	6	5
SnUK 17	0.016	0.012	0.014	0.012	0.01	0.009	0.01	0.014	0.006	0.013	0.007	0.006	0.01	0.007		3	2	6	5
SnUK 18	0.014	0.01	0.012	0.01	0.009	0.007	0.009	0.012	0.004	0.011	0.006	0.004	0.008	0.005	0.005		1	5	4
SnUK 19	0.012	0.009	0.01	0.009	0.007	0.005	0.007	0.01	0.002	0.009	0.004	0.002	0.006	0.003	0.003	0.002		4	3
SnUK 20	0.012	0.009	0.01	0.009	0.007	0.005	0.007	0.01	0.007	0.015	0.006	0.007	0.008	0.01	0.01	0.009	0.007		1
SnUK 21	0.01	0.007	0.009	0.007	0.005	0.003	0.005	0.009	0.006	0.013	0.004	0.006	0.006	0.009	0.009	0.007	0.005	0.002	

Table 3: Uncorrected p – distances between sequences of unique ITS2 haplotypes between *R. balthica* and *R. peregra* represented as p – distance in the bottom left and numbers of nucleotide substitutions between sequences in the top right

	HQ003232 *R. balthica*	HE573086 *R balthica*	AJ319634 *R. peregra*	KF887039 *R. peregra*	SnUK 9	SnUK 18
HQ003232 *R. balthica*		2	3	2	2	2
HE573086 *R. balthica*	0.006		3	2	2	2
AJ319634 *R. peregra*	0.009	0.008		2	3	3
KF887039 *R. peregra*	0.006	0.005	0.005		2	2
SnUK 9	0.006	0.005	0.008	0.005		2
SnUK 18	0.006	0.005	0.008	0.005	0.005	

Table 4: Genetic diversity and estimates of nucleotide substitution saturation between *Radix* species using Cox1 and ITS2. A) Genetic diversity where S = number of segregating sites; π = nucleotide diversity; K = number of observed pairwise differences; Tv = mean

transversions; Ts = mean transitions; *p*-distance = uncorrected pairwise distance. B) Nucleotide substitution saturation as described by Xia et al. (2003) where significant Iss < Iss.c = little saturation; non-significant Iss < Iss.c = little saturation; significant Iss > Iss.c = useless sequences; non-significant Iss > Iss.c = poor for phylogenetic analysis

A) Genetic Diversity

Molecular marker	No. sites	S	π	k	Tv	Ts	*p*-distance
Cox1	572	175	0.10204 (SD±0.00497)	49.18512	22.99734 (SD±15.4752)	28.02131 (SD±14.94986)	0.09806 (SD±0.05683)
ITS2	483	67	0.03890 (SD±0.00681)	6.84577	12.313 (SD±10.56)	13.935 (SD±10.95)	0.076 (SD±0.061819)

B) Substitution saturation output

Molecular marker	Proportion of Invariant Site	Iss	Iss.c	DF	*P*-value
Cox 1	0.55413	0.318	0.740	214	0.000
ITS2	0.08648	0.0725	0.71225	160	0.000

Table 5: Genetic diversity of *Radix auricularia* and *R. balthica* sampled in this study and cox1 comparisons with population data from other studies. S = segregating sites; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity; K = number of observed pair wise differences.

	No. seq	S	h	Hd	π	K
Radix auricularia sampled in this study Cox1	8	9	5	0.857	0.00687	3.92857

				(SD±0.108)	(SD±0.00191)	
ITS2	8	1	2	0.571 (SD±0.094)	0.00142 (SD±0.00023)	0.57143
Radix balthica sampled in this study						
Cox1	13	8	6	0.641 (SD±0.150)	0.00410 (SD±0.00102)	2.20513
ITS2	13	2	3	0.5 (SD±0.136)	0.00137 (SD±0.00043)	0.53846
Cox1 comparisons with data from populations generated by other studies						
R. auricularia	33	88	23	0.960 (SD±0.022)	0.03289 (SD±0.00761)	14.07765
R. balthica	55	105	34	0.969 (SD±0.013)	0.01487 (SD±0.00447)	7.51044

Figure1

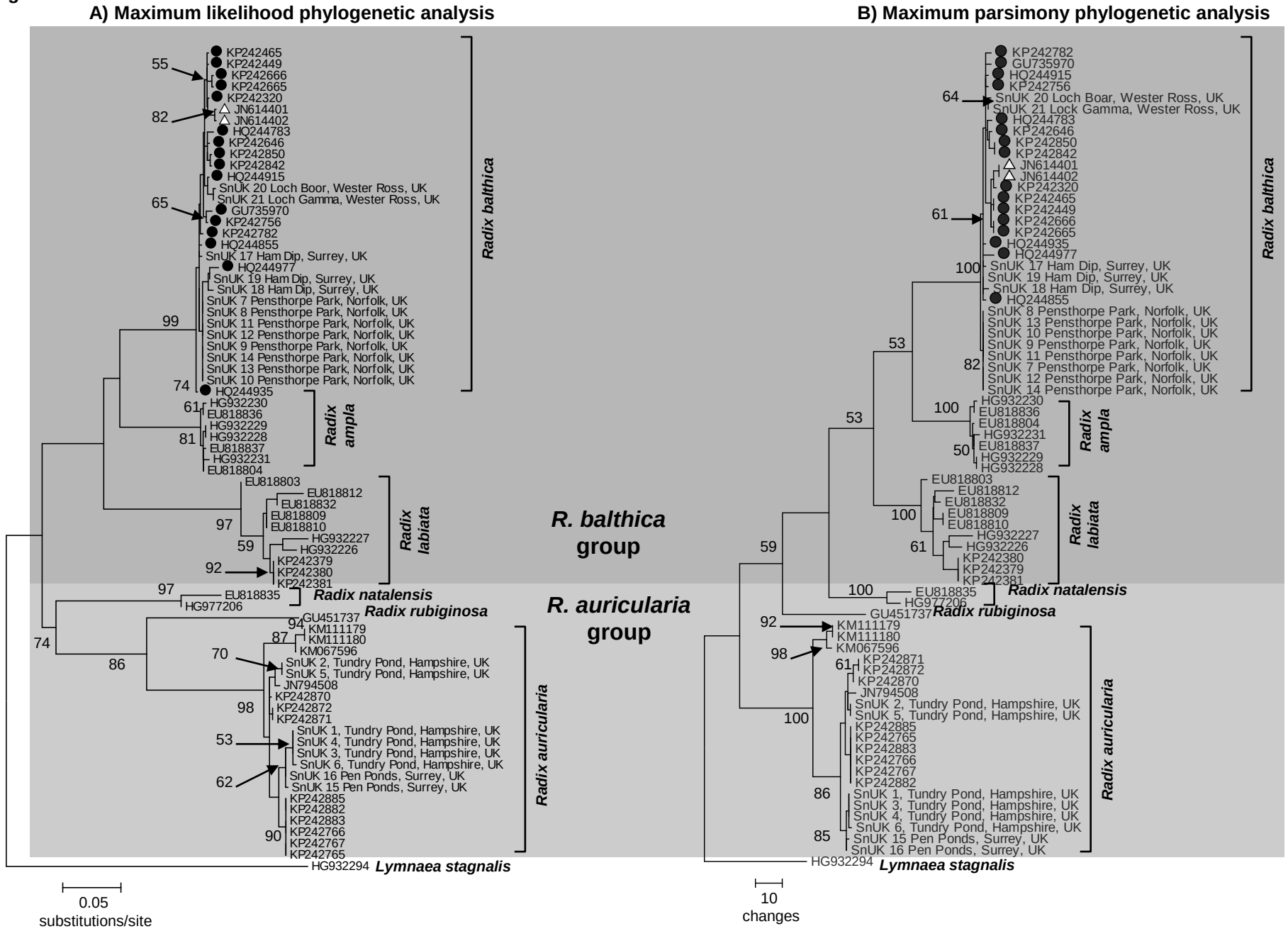

A) Maximum likelihood phylogenetic analysis
B) Maximum parsimony phylogenetic analysis
R. balthica group
R. auricularia group
Radix balthica
Radix ampla
Radix labiata
Radix natalensis
Radix rubiginosa
Radix auricularia
Lymnaea stagnalis
KP242465
KP242449
KP242666
KP242665
KP242320
JN614401
JN614402
HQ244783
KP242646
KP242850
KP242842
HQ244915
SnUK 20 Loch Boor, Wester Ross, UK
SnUK 21 Loch Gamma, Wester Ross, UK
GU735970
KP242756
KP242782
HQ244855
SnUK 17 Ham Dip, Surrey, UK
HQ244977
SnUK 19 Ham Dip, Surrey, UK
SnUK 18 Ham Dip, Surrey, UK
SnUK 7 Pensthorpe Park, Norfolk, UK
SnUK 8 Pensthorpe Park, Norfolk, UK
SnUK 11 Pensthorpe Park, Norfolk, UK
SnUK 12 Pensthorpe Park, Norfolk, UK
SnUK 9 Pensthorpe Park, Norfolk, UK
SnUK 14 Pensthorpe Park, Norfolk, UK
SnUK 13 Pensthorpe Park, Norfolk, UK
SnUK 10 Pensthorpe Park, Norfolk, UK
HQ244935
HG932230
EU818836
HG932229
HG932228
EU818837
HG932231
EU818804
EU818803
EU818812
EU818832
EU818809
EU818810
HG932227
HG932226
KP242379
KP242380
KP242381
EU818835
HG977206
GU451737
KM111179
KM111180
KM067596
SnUK 2, Tundry Pond, Hampshire, UK
SnUK 5, Tundry Pond, Hampshire, UK
JN794508
KP242870
KP242872
KP242871
SnUK 1, Tundry Pond, Hampshire, UK
SnUK 4, Tundry Pond, Hampshire, UK
SnUK 3, Tundry Pond, Hampshire, UK
SnUK 6, Tundry Pond, Hampshire, UK
SnUK 16 Pen Ponds, Surrey, UK
SnUK 15 Pen Ponds, Surrey, UK
KP242885
KP242882
KP242883
KP242766
KP242767
KP242765
HG932294
0.05
substitutions/site
10
changes
Key: Radix balthica Radix peregra

Figure2

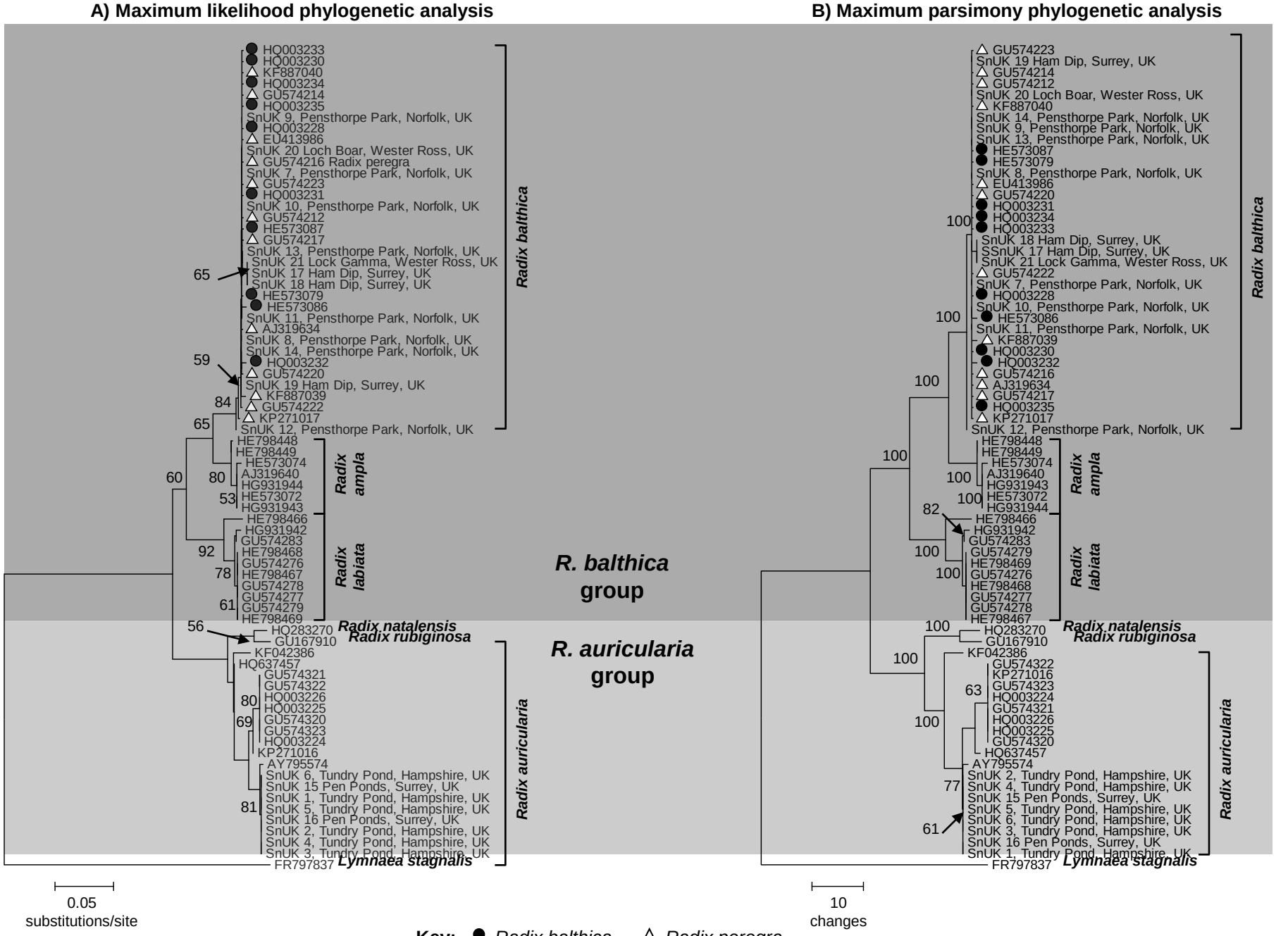

A) Maximum likelihood phylogenetic analysis
B) Maximum parsimony phylogenetic analysis
R. balthica group
R. auricularia group
Radix balthica
Radix ampla
Radix labiata
Radix natalensis
Radix rubiginosa
Radix auricularia
Lymnaea stagnalis
0.05 substitutions/site
10 changes
Key: Radix balthica Radix peregra

Figure3

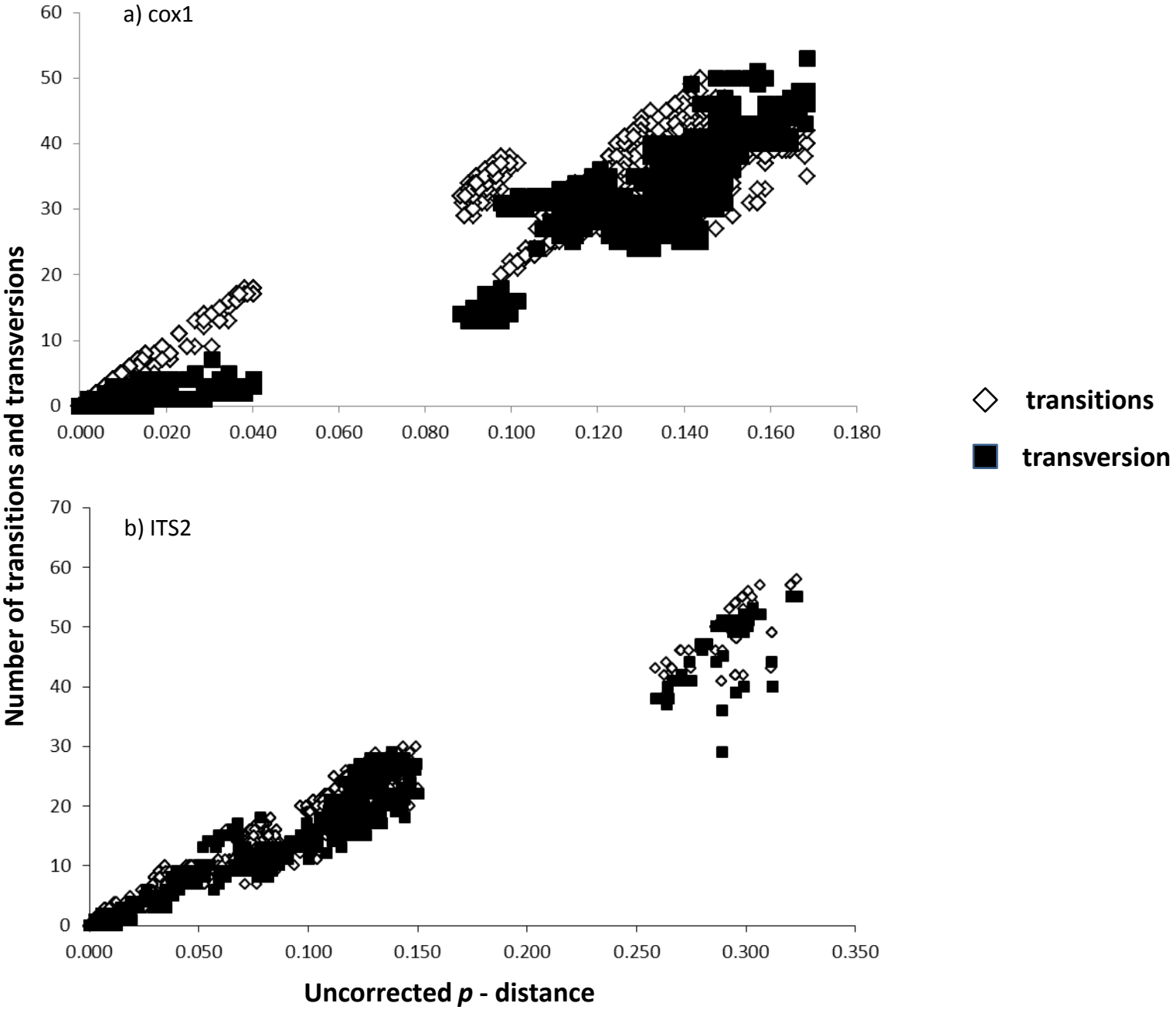

a) cox1
b) ITS2
Number of transitions and transversions
Uncorrected p - distance
transitions
transversion
0
10
20
30
40
50
60
70
0.000
0.020
0.040
0.060
0.080
0.100
0.120
0.140
0.160
0.180
0.050
0.150
0.200
0.250
0.300
0.350

Figure4

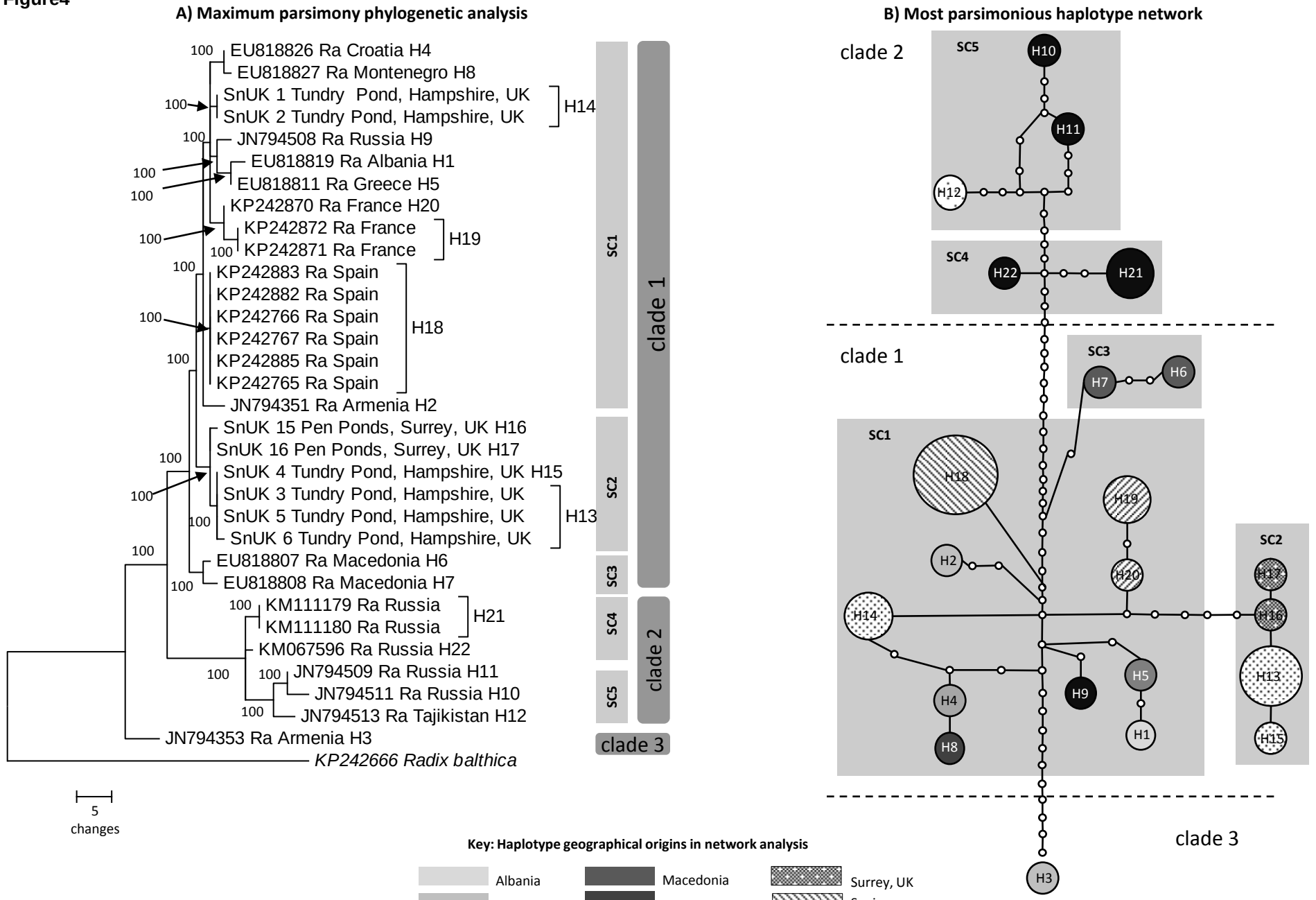
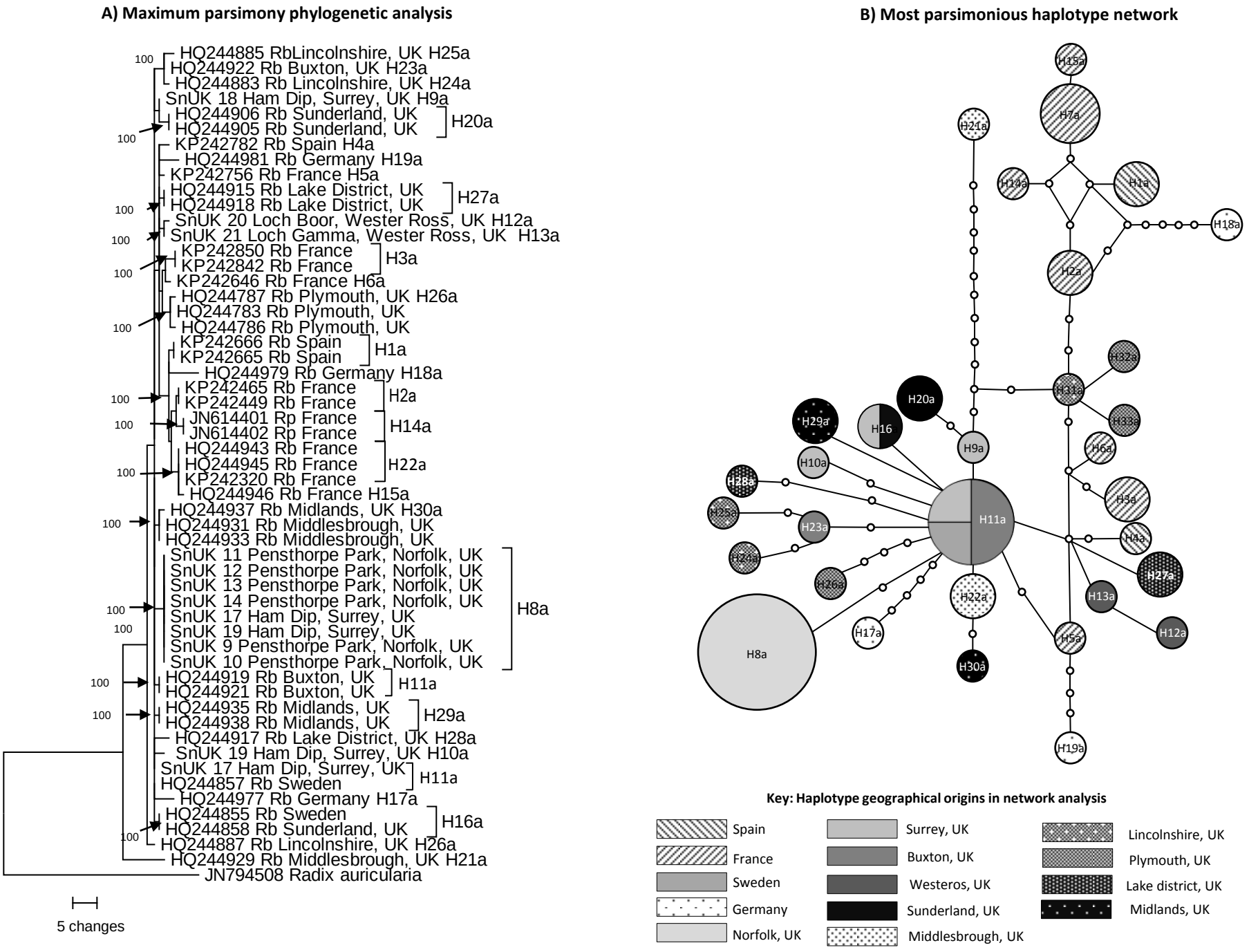

A) Maximum parsimony phylogenetic analysis
100 EU818826 Ra Croatia H4
EU818827 Ra Montenegro H8
SnUK 1 Tundry Pond, Hampshire, UK
SnUK 2 Tundry Pond, Hampshire, UK
H14
JN794508 Ra Russia H9
EU818819 Ra Albania H1
EU818811 Ra Greece H5
KP242870 Ra France H20
KP242872 Ra France
KP242871 Ra France
H19
KP242883 Ra Spain
KP242882 Ra Spain
KP242766 Ra Spain
KP242767 Ra Spain
KP242885 Ra Spain
KP242765 Ra Spain
H18
JN794351 Ra Armenia H2
SnUK 15 Pen Ponds, Surrey, UK H16
SnUK 16 Pen Ponds, Surrey, UK H17
SnUK 4 Tundry Pond, Hampshire, UK H15
SnUK 3 Tundry Pond, Hampshire, UK
SnUK 5 Tundry Pond, Hampshire, UK
SnUK 6 Tundry Pond, Hampshire, UK
H13
EU818807 Ra Macedonia H6
EU818808 Ra Macedonia H7
KM111179 Ra Russia
KM111180 Ra Russia
H21
KM067596 Ra Russia H22
JN794509 Ra Russia H11
JN794511 Ra Russia H10
JN794513 Ra Tajikistan H12
JN794353 Ra Armenia H3
KP242666 Radix balthica
SC1
SC2
SC3
SC4
SC5
clade 1
clade 2
clade 3
5
changes
B) Most parsimonious haplotype network
clade 2
clade 1
clade 3
SC5
SC4
SC3
SC1
SC2
H10
H11
H12
H22
H21
H7
H6
H18
H19
H2
H20
H14
H17
H16
H13
H15
H5
H9
H4
H8
H1
H3
Key: Haplotype geographical origins in network analysis
Albania
Armenia
Croatia
Greece
Macedonia
Montenegro
Tajikistan
Hampshire, UK
Surrey, UK
Spain
France
Russia

Figure5


A) Maximum parsimony phylogenetic analysis
HQ244885 RbLincolnshire, UK H25a
HQ244922 Rb Buxton, UK H23a
HQ244883 Rb Lincolnshire, UK H24a
SnUK 18 Ham Dip, Surrey, UK H9a
HQ244906 Rb Sunderland, UK
HQ244905 Rb Sunderland, UK
H20a
KP242782 Rb Spain H4a
HQ244981 Rb Germany H19a
KP242756 Rb France H5a
HQ244915 Rb Lake District, UK
HQ244918 Rb Lake District, UK
H27a
SnUK 20 Loch Boor, Wester Ross, UK H12a
SnUK 21 Loch Gamma, Wester Ross, UK H13a
KP242850 Rb France
KP242842 Rb France
H3a
KP242646 Rb France H6a
HQ244787 Rb Plymouth, UK H26a
HQ244783 Rb Plymouth, UK
HQ244786 Rb Plymouth, UK
KP242666 Rb Spain
KP242665 Rb Spain
H1a
HQ244979 Rb Germany H18a
KP242465 Rb France
KP242449 Rb France
H2a
JN614401 Rp France
JN614402 Rp France
H14a
HQ244943 Rb France
HQ244945 Rb France
KP242320 Rb France
H22a
HQ244946 Rb France H15a
HQ244937 Rb Midlands, UK H30a
HQ244931 Rb Middlesbrough, UK
HQ244933 Rb Middlesbrough, UK
SnUK 11 Pensthorpe Park, Norfolk, UK
SnUK 12 Pensthorpe Park, Norfolk, UK
SnUK 13 Pensthorpe Park, Norfolk, UK
SnUK 14 Pensthorpe Park, Norfolk, UK
SnUK 17 Ham Dip, Surrey, UK
SnUK 19 Ham Dip, Surrey, UK
SnUK 9 Pensthorpe Park, Norfolk, UK
SnUK 10 Pensthorpe Park, Norfolk, UK
H8a
HQ244919 Rb Buxton, UK
HQ244921 Rb Buxton, UK
H11a
HQ244935 Rb Midlands, UK
HQ244938 Rb Midlands, UK
H29a
HQ244917 Rb Lake District, UK H28a
SnUK 19 Ham Dip, Surrey, UK H10a
SnUK 17 Ham Dip, Surrey, UK
HQ244857 Rb Sweden
H11a
HQ244977 Rb Germany H17a
HQ244855 Rb Sweden
HQ244858 Rb Sunderland, UK
H16a
HQ244887 Rb Lincolnshire, UK H26a
HQ244929 Rb Middlesbrough, UK H21a
JN794508 Radix auricularia
100
5 changes
B) Most parsimonious haplotype network
H15a
H7a
H21a
H14a
H1a
H18a
H2a
H32a
H31a
H20a
H29a
H16
H33a
H9a
H6a
H10a
H28a
H3a
H25a
H23a
H11a
H4a
H24a
H27a
H26a
H22a
H13a
H17a
H5a
H12a
H8a
H30a
H19a
Key: Haplotype geographical origins in network analysis
Spain
France
Sweden
Germany
Norfolk, UK
Surrey, UK
Buxton, UK
Westeros, UK
Sunderland, UK
Middlesbrough, UK
Lincolnshire, UK
Plymouth, UK
Lake district, UK
Midlands, UK

Highlights

- Shows that *Radix peregra* is not responsible for the transmission of infection in the UK thanks to molecular techniques accurately identifying *"R. peregra"* to be either *Radix auricularia* or *Radix balthica*
- Provides a detailed comparison of the use of cox1 and ITS2 for the identification of snails of medical and veterinary and provides recommendations for their use
- Provides the first evolutionary insights into the relationship between UK and European populations of *Radix* species in relation to disease transmission