Mitochondrial variation of the caddisflies Apatania zonella and Potamophylax cingulatus

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1. Abstract

This project is a study of two different species of Trichoptera: *Apatania zonella* and *Potamophylax cingulatus. A. zonella* is a circumpolar species which lives at high latitudes, in cold clear-water, streams, lakes and marshes. *P.cingulatus* is widely distributed in rivers in northern and central Europe. The aim of the study is to find the origin of the two Icelandic Trichoptera species, by comparing them with conspecifics from the neighbouring countries. Samples were obtained from Norway, Britain, Alaska, Greenland, Iceland and the Faeroes Islands.

To study the genetic relationships, I sequenced a 1098 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, a commonly used marker for such studies.

The results show different result for the two species. The genealogy of *A. zonella* is clustered in several monophyletic groups representing geographical areas, and which are well differentiated. The population in Iceland is an exception, showing and admixture of two well differentiated lineages which have colonized Iceland, one from North America and the other from mainland Europe. *P. cingulatus* however does not show any variation within Iceland, its variation reflects a recent colonization, must likely from Britain via Faeroes Islands.

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5. Introduction

Phylogeography is the study of the relationships between history, ecology and biotic diversification, combining information from population genetics, phylogenetics, geoclimatic history, paleontology, population biology, molecular evolution and historical biogeography in order to understand the causes for the contemporary geographic distributions of individuals. In all these fields, glaciations have had large impact of the results obtained, as they have repeatedly induced shifts in the distribution of species and extinction of populations and genotypes (Alsos et al., 2005).

There have been five known Ice Ages in the Earth's history. The Cenozoic is characterized by a significant cooling of the high latitudes, which ultimately led to the Quaternary situation, the last period of glaciations, which was an extensive glaciations in both hemispheres interrupted by warmer interglacial phases (Geirsdóttir et al., 2007). The Quaternary period started about 2.4Myr ago and ended about 0.01Myr ago, the ice sheets advanced and receded with a roughly 41,000-yr cycle; thereafter they have followed a 100-kyr cycle and become increasingly dramatic (Hewitt, 2000). Quaternary climatic oscillations, punctuated by the Pleistocene glaciations, caused massive changes to the distributions of species in the Palaearctic (Hewitt, 2000, 2004; Schmitt, 2007), demography and, thus, evolution of species (Wahlberg and Saccheri, 2007). As a consequence species went extinct over large parts of their range, some dispersed to new locations and some survived in refugia and then expanded again (Hewitt, 2000). Repeated cycles of demographic contraction and expansion, into and out of pockets of climatically protected regional refugia, combined with individual ecologies, has led to varied patterns of present day phylogeographic concordance among species (Hewitt, 1999). Isolation among refugial populations promotes genetic and phenotypic differentiation as a result of independent adaptation to local environments and genetic drift, with consequences for reproductive isolation between discrete refugial lineages and the creation of hybrid contact (Hewitt, 1999). The effects of the Ice Age on species ranges varies with latitude and topography (Hewitt, 2004); thus, it is now well established that in the northern hemisphere many temperate species retreated from large continental areas during the height of the last glaciations and were only able to survive in sheltered refugia, which provided suitable conditions. However, controversy and uncertainly remain regarding the number and location of glacial refugia that contributed to modern populations (Kotlik et al., 2006).

Iceland is the largest landmass (103,000km²) in the central North Atlantic (Geirsdóttir et al., 2009), where glaciations have been preserved, along with Greenland, in the North Atlantic. This inimitable geographic and climatic position enhances the island's importance in glacial geological and palaeoclimatic research (Geirsdóttir et al., 2007). The mapping of glacial deposits in Iceland has also revealed a progressive spread of the Icelandic ice sheet through time, making it possible to draw inferences about the intensities of individual glaciations, changes in

topography and landscape evolution since the initiation of glaciations in Iceland (Geirsdóttir et al., 2007). Islands are a microcosm of continents insofar as the ecological and evolutionary processes are identical in both (Vitousekm et al., 1995); however, attributes such as, relative size, isolation, geological history, taxonomic diversity, endemism, colonization patterns and varying levels of human contact make them different from continental regions.

In this study I look at the phylogeography of caddisflies (Trichoptera), which are the most species-rich group of aquatic insect and are adapted to a wide range of specialized habitats. Within Trichoptera I have chosen two different species: *Apatania zonella* (Ztetterstedt, 1840) and *Potamophylax cingulatus* (Stephen, 1837). Caddisflies larvae are important and beneficial component of the trophic dynamics and energy flows in the lakes, rivers, and streams they inhabit (Resh and Rosenberg, 1984); and they are considered among the most useful and important aquatic organisms for monitoring the environmental impact that these surface waters receive (Holzenthal et al., 2007).

- *A.zonella* lives today in high densities close to glaciers, in cold clear-water streams and lakes, where estimated summer water temperatures may be between 10 and 12°C. The modern distribution is circumpolar, including northern Russia, the Baltic region, Finland, northern Sweden, Norway, Spitbergen, Iceland, Greenland, and the northern part of North America (Botosaneanu and Malicky, 1978; Solem and Andersen, 1996; Wiggins, 1996, Solem and Birks, 2000).

- *P.cingulatus* larvae are found in lakes, streams, and marshes; and also is the dominant group at higher latitudes and elevations, over whole Europe and European Russia (Holzenthal, et al., 2007). This species has just recently colonized Iceland or around 1975.

The distribution of population-level genetic diversity of a species is a product of the species' life history and dispersal patterns, geographic history, climatic history and chance (Avise, 2007; Pauls et al., 2009). Species responses to climate change may vary for organisms depending on their level of cold-tolerance and dispersal capabilities (Schönswetter et al., 2004; Deffontaine et al., 2005; Pinceel et al., 2005). In the boreal regions, the environmental conditions are often very severe, and make survival and reproduction more difficult. Fluctuations of population size are common in many boreal species that can have regular cycles or irregular population outbreaks. The effect of such fluctuations and of local extinctions and recolonizations on genetic differentiation is complicated (McCauley et al., 1995; Barton and Withlock, 1996) but they increase the effect of drift and can, under some combinations of parameters, therefore lead to increased genetic differentiation (Pamilo and Savolainen, 1999).

The time frame of the development of boreal ecosystems is generally considered to be too short for speciation, because the ice only receded ten thousand years ago and most population genetic processes require long times to reach equilibrium (Pamilo and Savolainen, 1999). As a result of the short evolutionary time, species are characterized by shallow genealogies, clear boundaries and little variation. We know caddisflies' refugias were in refugial areas in Europe (Previšić et al., 2009) and, with the recession of the ice sheet, they started to colonize new boreal countries as Greenland, Iceland, Norway or Alaska. The time since colonization can vary between each country, can find old or recent populations because of new migrations and the dispersal patterns that they had.

The aim of the study is to study the origin of the Icelandic caddisflies by comparing its mitochondrial DNA (mtDNA) sequence variation with conspecifics from other neighboring countries.

To answer these questions I have used mtDNA because it does not recombine and its inheritance is only maternal. It also has a relative fast mutation rate and has a lower effective population size than nuclear markers, which results in significant variation in mtDNA sequences between species and, in principle, a comparatively small variance within species. However, the main arguments made against using mtDNA is that its maternally inheritance does not represent the "true" genomic inheritance of an organism (Rubinoff and Holland, 2005), possibly due to sex dependent migration. MtDNA has also been shown to be affected by occurrences of hybridization (Melo-Ferreira et al., 2005), male-killing (Johnstone and Hurst, 1995), cytoplasmic incompatibility-inducing symbionts (Hurst and Jiggins, 2005), horizontal genes transfer (Croucher et al., 2004), or other "reticulate" evolutionary phenomena in a lineage which can lead to misleading results. Its rapid mutation size (in animals) along with the low effective population size, makes mtDNA useful for assessing genetic relationships of individuals or groups within a species and also for identifying and quantifying the phylogeny among different species that are closely or moderately-closely related. Comparison of the mtDNA sequences from different individuals or species allows construction of a network of the relationships among the sequences, which provides an estimate of the relationships among the individuals or species from which the mtDNAs were taken. DNA barcoding employs sequence diversity in short standardized gene regions to aid species identification in large assemblages of life (Ratnasingham and Hebert, 2007). Within the mtDNA, we look at sequence variation of a part of the cytochrome c oxidase subunit 1 (CO1) gene because this region has been accepted as a practice, standardized species-level barcode for animals, because it:

- contains significant species-level genetic variability and divergence,
- possess conserved flanking sites for developing universal PCR primers for wide taxonomic application,
- has a short sequence length so as to facilitate current capabilities of DNA extraction and amplification (Ratnasingham and Hebert, 2007).

6. Material and methods

6.1. Samples

Thirty four samples belonging to two different species were studied in this project.

The samples were from Norway, Britain, Iceland, Alaska and Greenland.

The species were Apatania zonella and Potamophylax cingulatus. The samples of A. zonella were from Norway, Greenland, Alaska and Iceland; the samples of P. cingulatus were from Norway and Britain. Information on samples of *P. cingulatus* from Iceland and The Faeroes Islands were included in the study.

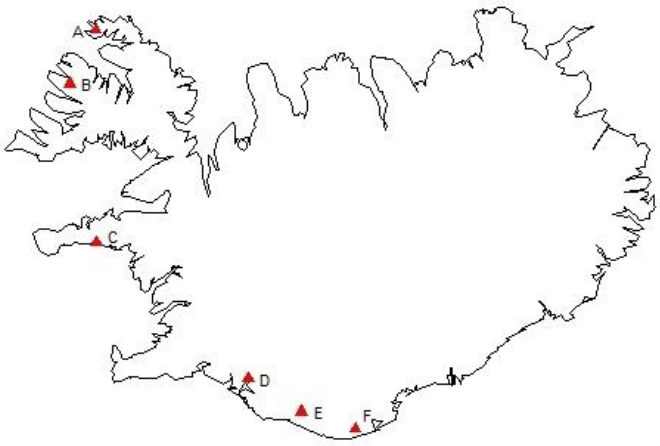


Figure 1. Samples sites of *A.Zonella* in Iceland: (A) Aðalvík, (B) Bjarnadalsá, (C) Staðará, (D) Ytri Rangá, (E) Hofsá, (F) Blautakvísl.

Figure 2. Samples sites of *A.zonella* in Greenland: (A) Brattahlid, (B) Narsarsuaq, (C) Gardar.

Figure 3. Samples site of *A.zonella* in Alaska: (A) Galbraith lake

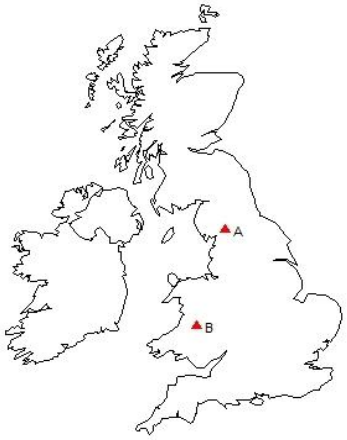


Figure 4. Samples sites of *P.cingulatus* in Britain: (A) Cumbria, (B) Wales.

Figure 5. Samples site of *A.zonella* in Norway: (A) Dofrefjell; and samples site of *P.cingulatus* in Norway: (B) Hördaland

6.2. Laboratory protocols

6.2.1. DNA extraction

DNA was extracted from dissected flies' larvae or whole individual using chelex 6% and proteinase K. Chelex protects the samples from DNAases that might remain and contaminate the samples; and proteinase K is used to remove all the proteins by digestion.

The samples are mixed with 350µl chelex 6% and 3.5µl proteinase K; then they were kept at 65°C for 3 hours, warmed to 100°C for 9 minutes and centrifugate 2 minutes at 13 krpm. After that they were stored in the fridge.

Fifteen samples from Alaska and Greenland were extracted and other nineteen samples from Iceland, Norway and Britain were already extracted.

6.2.2. PCR: Polymerase Chain Reaction

Polymerase Chain Reaction is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands of copies of a particular DNA sequence.

A fragment of the COI mitochondrial (mtDNA) gene was targeted. For this amplification two pairs of primers (see table 1) were used.

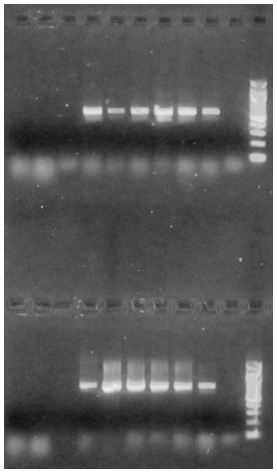
Figure 6. CO1 gene representation with primers. PCR amplification were done in two separate reactions, one using LCO1980 and HCO2198 and the other Jerry and S20.

PCR were performed in 10µl volumes containing 4.82µl distilled water, 0.75µl dNTP's (2mM), 1.15µl tween 20 (1%), 1µl taq buffer (10x), 0.5µl BSA (10mg/ml), 0.34µl of each primer (10pM)(forward and reverse), 0.1µl taq polymerase and 1µl of template DNA (ca 10-200ng).

PCR conditions for CO1 fragment involved initial denaturing of 5 minutes at 94°C, then 40 cycles of 20 seconds at 94°C, 45 seconds at 49°C, 1 minute at 72°C and 6 minutes of extension at 72°C.

Electrophoresis was used in order to evaluate the result from the PCR. The concentration of the agarose gel is 1.5%, and the samples were run for 20 minutes at 100mV. The PCR products were visualized taking picture of the agarose gel under UV light.

(A)



(B)

Figure 7. Agarose gel electrophoresis of DNA fragments generated by amplification of *A.zonella* samples from Iceland using (A) Jerry/S20 and (B) LCO/HCO

6.2.3. Exosap reaction

Once we know our DNA has been amplified successfully, we do the exosap reaction, which is used for remove the unwanted dNTP's and primers, and also residual single-stranded primers and extraneous single-stranded DNA produced by the PCR.

Exosap mix containing 0,75µl ddH_2O, 0,7µl Antarctic phosphatase buffer, 0,5µl Antarctic phosphatase and 0,05µl ExoI, which is adding to 6µl PCR product.

The mix was warmed in PCR machine for 30 minutes at 38°C for treatment and then 15 minutes at 80°C to inactive enzymes.

6.2.4. Sequencing

Big Dye is a ready reaction mix which contains all the components necessary to the sequencing reaction: Amplitaq DNA polymerase FS, dNTPs, ddNTPsDye terminators pyrophosphatase, Buffer and $MgCl_2$.

Cycle sequencing reaction mix contained 2.65µl dd H_2O, 1.75µl buffer, 1µl big dye, 1.6µl primer and 3µl PCR product. The samples were sequenced using the same forward and reverse primers used for PCR but at 10 folder lower concentration (1pM).

Table 1. Primers used in PCR and sequencing.

Primer	Direction	Sequence (5'–3')	Reference
LCO 1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
HCO 2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
Jerry	Forward	CAACATTTATTTTGATTTTTTGG	Simon et al. 1994
S20	Reverse	GGGAAAAAGGTTAAATTTACTCC	Simon et al. 1994

Sequence reaction involved 10 seconds at 96°C, 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 2 minutes at 60°C.

6.2.5. Ethanol precipitation

After sequencing, ethanol precipitation with glycogen is a method to purify or/and concentrate DNA from aqueous solutions.

The protocol:

- To each 5 - 10µl sequencing reaction add 50µl of a water NaOAc (0.3M) glycogen solution.
- Add 125µl cold (-20°C) 96% EtOH. Mix well by tipping back tubes back and down.
- Spin down at 4,000 rpm during 30 min at 0-4°C. Immediately pour off (most of) the precipitation mix.
- Wrap with 2 kim wipes, and spin down inverted at 300 rpm for 2 min at 0-4°C to remove the rest of the EtOH.
- Carefully, add 200µlof cold (-20°C) 70% EtOH.
- Spin at 4,000 rpm for 5 min at 0-4°C, immediately dump the EtOH and spin again inverted, covered with kin wipes, for 5 min at 300 rpm at 0-4°C.
- Let tubes air dry (in the dark) for 15 min to get rid of lost traces of EtOH (no pellet visible).
- Add 10µl HiDi, vortex and spin down.

The products were run on ABI Prism 3100 Genetic Analyser.

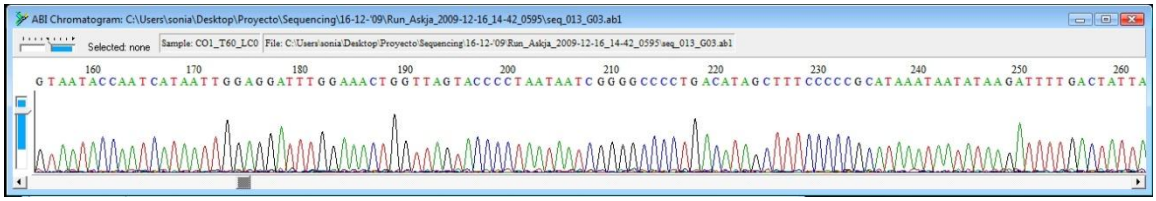


Figure 8. Electropherogram generated after sequencing.

6.3. Sequence variation

Thirty four individuals were sequenced for the length of 1200 base pairs.

For comparison, published sequences of two different species of Trichoptera were imported from Gen Bank: *Paraphlegopteryx morsei* (gb: AY536738.1) and *Lepidostoma arcuatum* (gb: AY536736.1). These sequences were used as an outgroup to root the phylogenetic trees.

The following computer programs were used:

- Chromas Lite (Technelysium Pty Ltd), to correct the sequences by comparing sequences with the electropherogram.

- BioEdit for read the sequences and align them to see easily segregation sites.
- R (R Development Core Team, 2008) to create the geographical maps (map package); and also to find the best-fitting model to construct the trees (ape package). The ape package was used to select the best evolutionary distance model among the sequences, applying the method developed by Posada (Posada and Crandall, 2001), and then using the maximum likelihood program Phyml (Guindom and Gascuel, 2003). The program finds the tree which maximizes the likelihood of the observed sequences.
- Network software to construct networks for analyzing the sequences relationships, the number of mutations and to visualize their geographical distribution.
- Mega (Tamura et al. 1993-2008) for analyze the sequences obtained by constructing a phylogenetic tree and see distance relations between species.

Phylogenetic trees were done with Mega using two different methods in order to study the phylogeny of *Apatania zonella* and *Potamophylax cingulatus*:

- Maximum likelihood, a powerful statistical method that seeks the tree that makes the data most likely. It tries to infer an evolutionary tree by finding that tree which maximizes the probability of observing the data (Hall, 2008).
- Neighbor-joining method, the most widely used distance method because it is fast and straightforward and has proven to be a reliable method. Its principle is to construct the tree by successive pairing of taxons (the neighbors): the pair that leads to the tree with the smallest total branch length is selected. The algorithm requires knowledge of the distance between each pair of taxa in the tree (Hall, 2008; Paradis, 2006).
- Maximum Parsimony, a method based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment (Hall, 2008).

The evolutionary models used in the trees were:

- Tamura-Nei, which is a model where both kinds of base transitions A-G and C-T have different rates and the base frequencies may be unequal (Paradis, 2006).
- The General Time-Reversible model in which all substitution rates are different, and the base frequencies may be unequal (Paradis, 2006).

Bootstrap test have been used to estimate the reliability of the trees.

7. Results

7.1. Haplotypes within *Apatania zonella* and *Potamophylax cingulatus*

Thirty four sequences were obtained for the CO1 gene; 1098 pair bases in length, and another sequence of *P. cingulatus* from Iceland and Faeroe Islands was used just to compare it with sequences from the others countries.

Table 2. Sequence variation in 1098 bp of the mtDNA COI gene. Comparison within and between species looking the variable sites, the synonymous and non-synonymous substitutions per site and the ratio between transitions and transversions.

	A. zonella	*P. cingulatus*	Between species
Ratio of nucleotide variable sites	54/1098	20/1098	219/1098
Ratio of amino acid variable sites	6/366	6/366	22/366
Synonymous changes	0.061	0.019	1.264
Non-synonymous changes	0.001	0.001	0.036
Non-syn./Syn.	0.0163	0.0526	0.0284
R= transition/transversion	12.751	5.148	1.026

Most nucleotide changes were synonymous (see table 2) and the same number of amino acid changes was observed within both species.

To determine the synonymous and non-synonymous substitutions rates I have used Nei-Gojobori method, which computes the number of synonymous and non-synonymous substitutions and the numbers of potentially synonymous and potentially non-synonymous sites (Nei and Gojobori, 1986). In this study, the non-synonymous sites are lower than the synonymous sites, which mean the variation in the amino acid sequence is not so high, and the protein has been conserved. Also, attending species, *P. cingulatus* has more rate changes than *A. zonella*; thus, *A. zonella* has more synonymous sites and *P. cingulatus* has more variable sequences. Figure 9 shows an example of the variable sites.

The transition/transversion ratio R is the ratio of the number of transitions to the number of transversions for a pair of sequences. R becomes 0.5 when there is no bias towards either transitional or transversional substitution because, when the two kinds of substitutions are equally probable, there are twice as many possible transversions as transitions. We can see in Table 2 that *A. zonella*'s ratio is higher than *P. cingulatus*, so there is a major bias towards transitional substitution. However this ratio is close to one between the species.

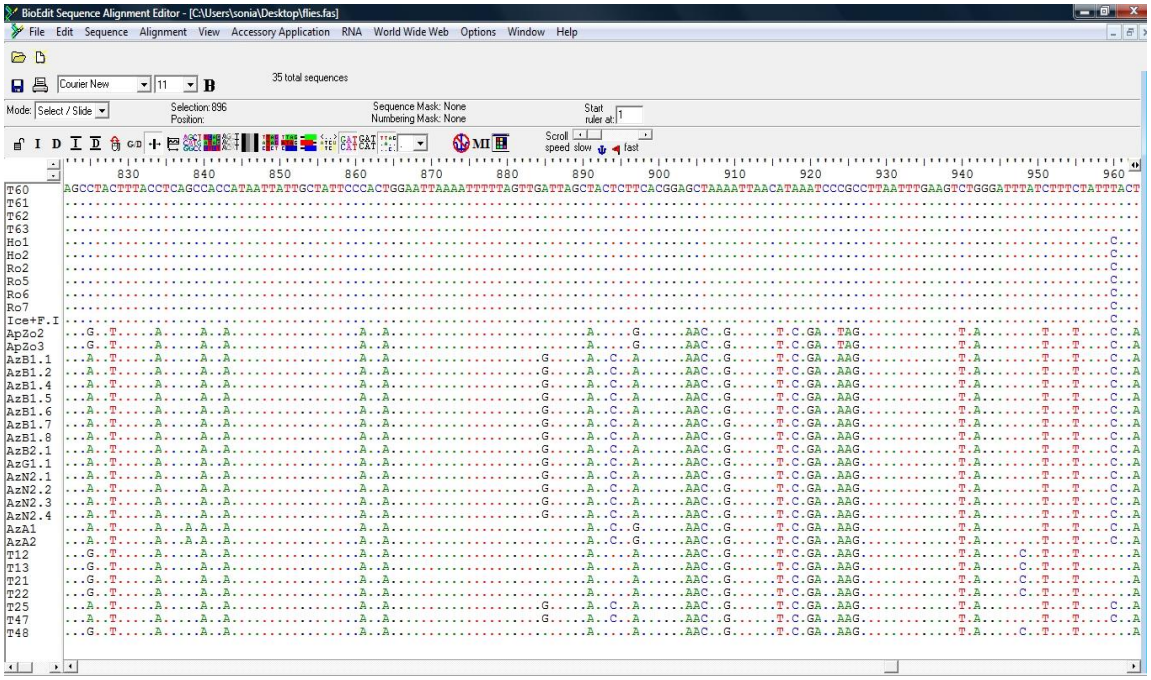


Figure 9. Variable sites in *Potamophylax cingulatus* and *Apatania zonella*.

Haplotype networks were constructed using Network software with sequences of individuals within *A. zonella* and *P. cingulatus* from different countries.

- *A. zonella*

Within twenty-four sequences obtained of *A. zonella*, eleven haplotypes (A – L) were distinguished. (See table 3 and Fig 9):

- Haplotypes A and B were found in sequences from Norway, one sequence in each haplotype.
- Haplotype C was found in three sequences from Iceland, but each from different locations.
- Haplotype E was only found in sequences from Alaska, two sequences with the same haplotype.
  Haplotype J was found in five sequences from Greenland, from two different places.
- Haplotype K was the most common, found in six sequences; five of them are from Greenland, but also from two different locations and the other one is from Iceland.
- Haplotypes D, E, G, H, I and L were just found in one sequence each one.

Table 3. List of samples and places where *A. zonella* were collected.

	Place in the tree	Place	Specie
ApZo2	B	Dofrefield, Norway	*A. zonella*
ApZo3	A	Dofrefield, Norway	*A. zonella*
AzA1	F	Galbraith lake, Alaska	*A. zonella*
AzA2	F	Galbraith lake, Alaska	*A. zonella*
T12	D	Aðalvik, Iceland	*A. zonella*
T13	C	Aðalvik, Iceland	*A. zonella*
T21	C	Hofsá, Iceland	*A. zonella*
T22	E	Ytri Rangá, Iceland	*A. zonella*
T25	L	Blautakvísl, Iceland	*A. zonella*
T47	K	Staðará, Iceland	*A. zonella*
T48	C	Bjarnadalsá, Iceland	*A. zonella*
AzB1.1	J	Brattahlid, Greenland	*A. zonella*
AzB1.2	K	Bratthalid, Greenland	*A. zonella*
AzB1.4	K	Brattahlid, Greenland	*A. zonella*
AzB1.5	J	Brattahlid, Greenland	*A. zonella*
AzB1.6	J	Brattahlid, Greenland	*A. zonella*
AzB1.7	K	Brattahlid, Greenland	*A. zonella*
AzB1.8	K	Brattahlid, Greenland	*A. zonella*
AzB2.1	J	Brattahlid, Greenland	*A. zonella*
AzG1.1	K	Gardar, Greenland	*A. zonella*
AzN2.1	J	Narsarsuaq, Greenland	*A. zonella*
AzN2.2	H	Narsarsuaq, Greenland	*A. zonella*
AzN2.3	I	Narsarsuaq, Greenland	*A. zonella*
AzN2.4	G	Narsarsuaq, Greenland	*A. zonella*

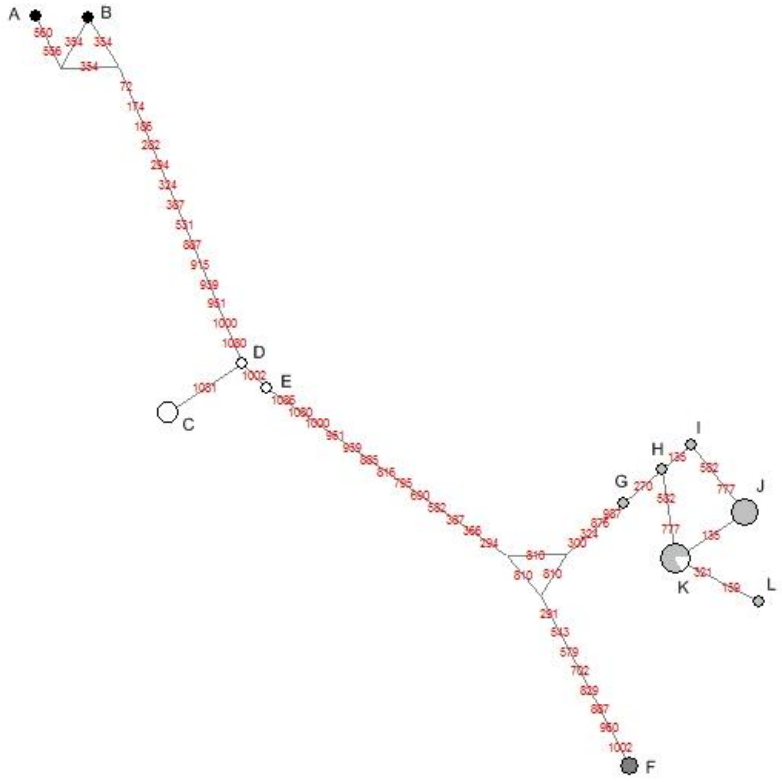


Figure 10. Tree of repartition of *Apatania zonella* with the software Network. Norway is in black, Iceland in white, Alaska in dark grey and Greenland in light grey.

Table 3. Haplotypes A – K are observed in the following flies:

- A: ApZo3
- B: ApZo2
- C: T13, T21, T48
- D: T12
- E: T22
- F: AzA1, AzA2
- G: AzN2.4
- H: AzN2.2
- I: AzN2.3
- J: AzB1.1, AzB1.5, AzB1.6, AzB2.1, AzN2.1
- K: T47, AzB1.2, AzB1.4, AzB1.7, AzB1.8, AzG1.1
- L: T25

- *P. cingulatus*

Eight haplotypes (A – H) among eleven sequences were distinguished. Most of haplotypes belong to a single sample; only haplotype E was found in four samples, being all of them from the same place in Norway (See table 4 and Fig. 10).

Table 4. List of samples and places where *P. cingulatus* were collected.

	Place in the tree	Place	Species
Ho1	G	Cumbria, Britain	*P. cingulatus*
Ho2	B	Cumbria, Britain	*P. cingulatus*
Ro2	C	Wales, Britain	*P. cingulatus*
Ro5	H	Wales, Britain	*P. cingulatus*
Ro6	A	Wales, Britain	*P. cingulatus*
Ro7	F	Wales, Britain	*P. cingulatus*
Ice	D	Iceland and Faeroe Islands	*P. cingulatus*
T60	E	Hördaland, Norway	*P. cingulatus*
T61	E	Hördaland, Norway	*P. cingulatus*
T62	E	Hördaland, Norway	*P. cingulatus*
T63	E	Hördaland, Norway	*P. cingulatus*

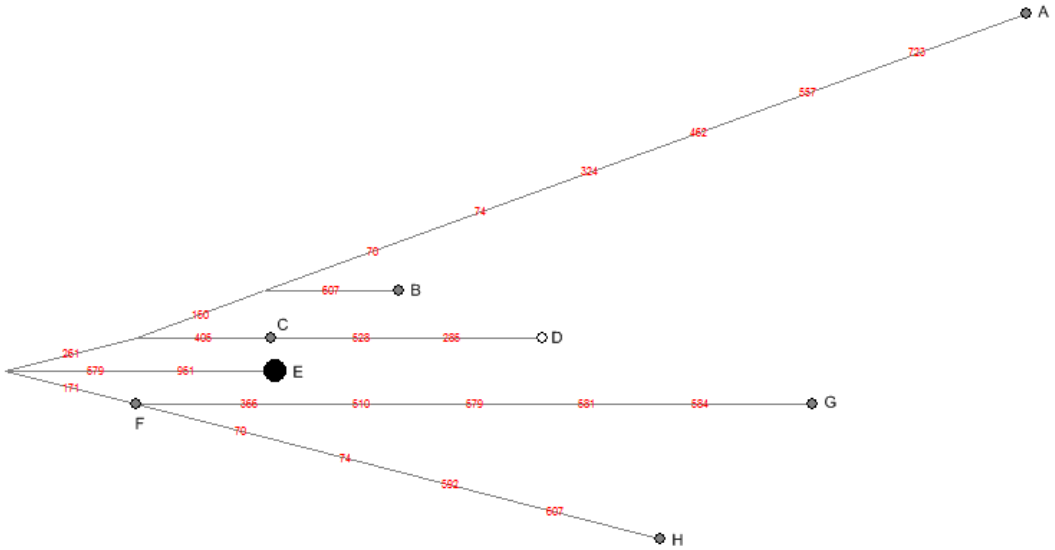


Figure 11. Tree of repartition of *Potamophylax cingulatus* with the software Network. Norway is in black, Britain in grey and Iceland + Faeroe Islands in white.

Table 4. Haplotypes A – H are observed in the following flies:

- A: Ro6
- B: Ho2
- C: Ro2
- D: Iceland + Faeroe Islands
- E: T60, T61, T62 and T63
- F: Ro7
- G: Ho1
- H: Ro5

7.2. Phylogenetic analysis

Figures 11 and 12 show the trees based on 1200 pair bases sequences from *A. zonella* and *P. cingulatus*. All of them were rooted with *Lepidostoma arcuatum* and *Paraphlegopteryx morsei* as an outgroup.

The best evolutionary distance is GTR+I+G ("General Timer Reversible"), in which the proportion of invariant sites is 0.464 and the gamma shape parameter is 1.207.

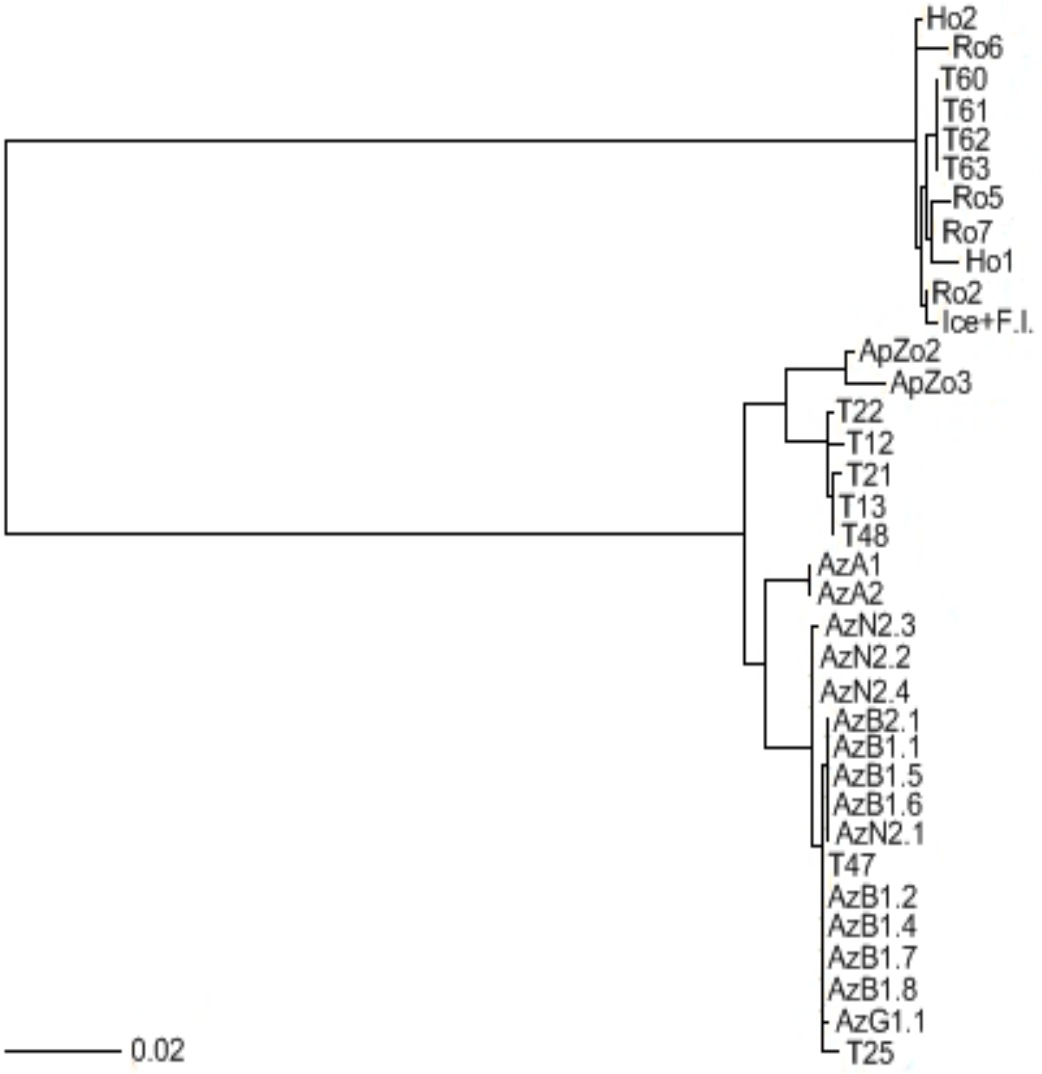


Figure 12. Phylogenetic tree based on CO1 mtDNA sequences of 1200 pair bases. The tree was constructed with R program using Maximun likelihood method and the "General Timer-Reversible" model.

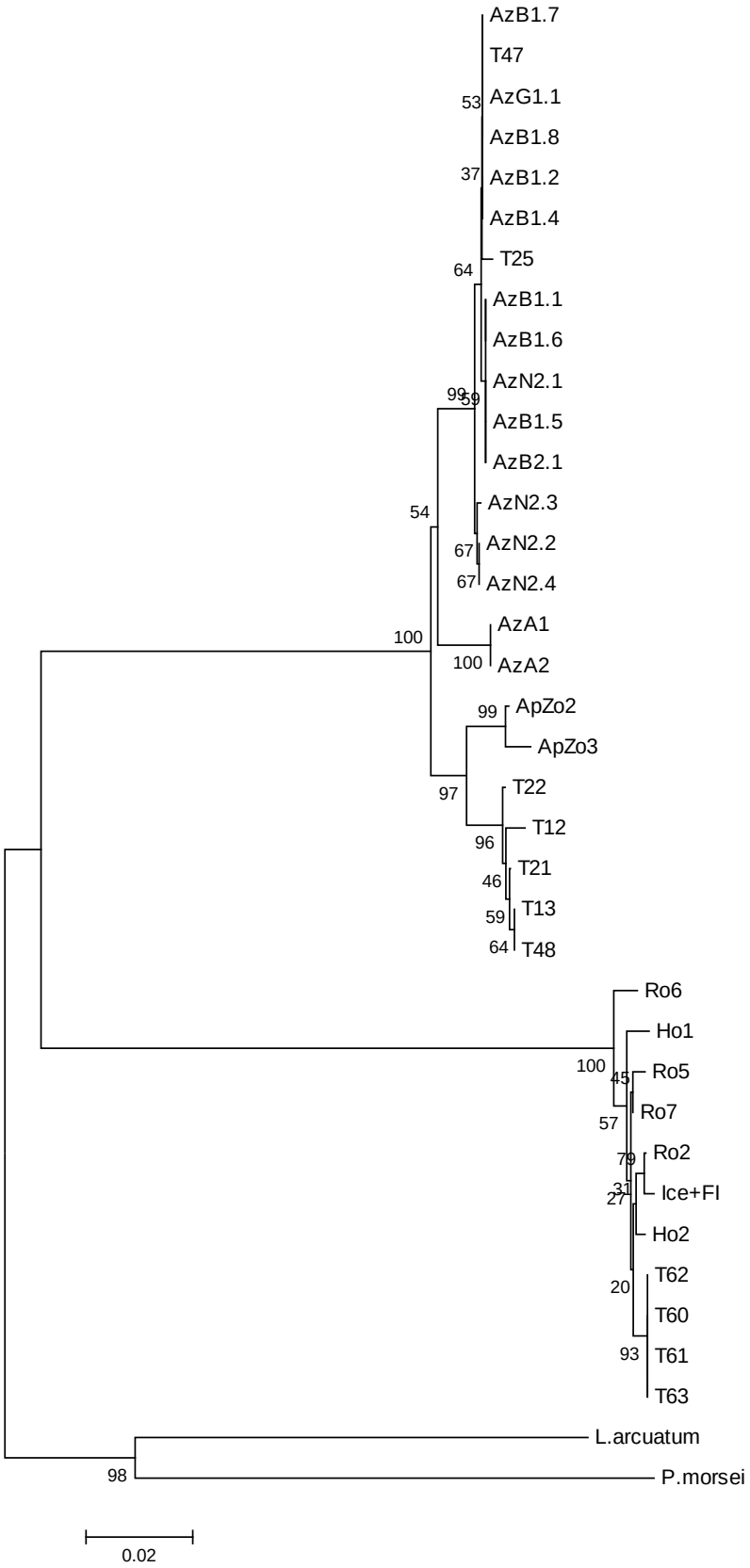


Figure 13. Phylogenetic tree based on CO1 mtDNA sequences of 1200 pair bases. The tree was constructed with Mega using the Neighbor-Joining method and Tamura-Nei model. Numbers on branches refer to the bootstrap obtained in percentages.

We can differentiate two big groups which correspond with both species: *A. zonella* and *P. cingulatus*. The numbers next to each division of the branches are the bootstrap values; most of them are lower than 70, which mean they are not really well supported, however, the values higher than 90 support:

- The division between both species.
- Within *P. cingulatus*, the division between the samples from Norway and the samples from Britain.
- Within *A. zonella*, the division between the samples from each country: Greenland, Alaska, Norway and Iceland.

The Maximum Parsimony method showed the same results as the Neighbor-Joining tree.

8. Discussion

8.1. Phylogenetic analysis

The phylogenetic trees show two species pretty well differentiated: *Apatania zonella* and *Potamophilax cingulatus*. Within *A. zonella* the monophyletic groups representing the geographic groups; and within *P. cingulatus* the samples are more closely-related between geographical zones. In both species we can see little variation, shallow genealogies and clear boundaries, influenced by the recent settlers about 10 kyr. ago. Nevertheless, the time of colonization varies and we can detect the movements towards other countries looking at the number of mutations.

The phylogenetic trees made with two different methods, Neighbor-Joining and Maximum Parsimony, corroborate the results obtained with the Network software about haplotypes within each species.

- ***A. zonella***

Populations within *A. zonella* are pretty well differentiated by countries. Within each country, we can find some differences:

- In Norway, two haplotypes were found, which differ by three mutations.
- In Alaska we just found one haplotype.
- In Greenland, where we find five haplotypes, two of them are found in five individuals from two different locations.
- In Iceland we also find five haplotypes, three of them quite similar between them and the other two are similar to Greenland samples.

The interpretation could be that ancestor of the flies survived in two different refugias, one in middle-south Europe and another one in America, acting as source populations which colonized Norway and Alaska respectively. From Norway they migrate into Iceland; and from Alaska towards Greenland. Two of the samples from Iceland are more similar to Greenland's flies than other Icelandic flies. Actually, one of them has the same haplotype as the five individuals obtain in two different places in Greenland, and the other one is different only by two mutations. The other flies from Iceland are most similar to the Norwegian flies. Thus the Icelandic population consists of flies originating both from Greenland or North-America, and from mainland Europe.

Within *A. zonella* there are two groups well differentiated, one from Iceland and Norway, and the other one from Alaska and Greenland; although two samples from Iceland are within the group from Greenland. The divergence within *A. zonella* is 0.017, which correspond almost 1 million years; and the divergence between groups is less, being 0.01 within Greenland and Alaska and 0.005 within Norway and Iceland.

- ***P. cingulatus***

Icelandic flies are genetically identical to the ones from Faeroe Islands indicating they have colonized Iceland via Faeroes. All flies from Norway have exactly the same haplotype, as we can see in Figure 11. The flies from Britain have different haplotypes, indicating that they arrived to Britain enough time ago to evolve and accumulate mutations or a larger population colonized Britain. A more extensive geographic sampling has also been done in Britain than in Norway. These haplotypes found in Iceland and the Faeroes is most similar to Ro2 found in Britain.

Nevertheless, all flies from Norway, Britain, Iceland and Faeroe Islands have a recent common ancestor, divergence is just 0.005, suggesting that all of them descend to the same population who may have survived in the same refugia during the Quaternary period.

8.2. General discussion

Within *P. cingulatus* we can see there is low genetic diversity, even between countries, indicating recent colonization. The reason is that geographical areas sampled at high latitudes were covered by an ice sheet repeatedly during the Quaternary period Flies couldn't colonize these territories until the recession of the glaciers occurred 10 kyr. ago and since then, flies migrated to new places.

Variation in the circumpolar species *A. zonella* is as may be expected in ring species. Ernst Mayr (1942) defined ring species the "perfect demonstration of speciation". In this case Iceland acts as a region or a clear hybrid zone where two different populations have arrived to Iceland from both ends of its range distribution; from Europe and from North-America. This study has been realized with a mitochondrial gene, which is maternally inherited. Although the sex ratio in *A. zonella* is highly skewed, about one percent or less is believed to be male we don't know whether rare sexual reproduction occur. To detect the mode of sexual reproduction it could be interesting to look at nuclear markers to know if the two populations in Iceland (the one from Norway and the other one from Greenland) and thus whether the two are interbreeding in Iceland.

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