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LIST OF ORIGINAL PUBLICATIONS

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I. **Laurimäe, T.,** Kinkar, L., Moks, E., Romig, T., Omer, R.A., Casulli, A., Umhang, G., Bagrade, G., Irshadullah, M., Sharbatkhori, M., Mirhendi, H., Ponce-Gordo, F., Soriano, S.V., Varcasia, A., Rostami-Nejad, M., Andresiuk, V., Saarma, U. (2018). Molecular phylogeny based on six nuclear genes suggests that *Echinococcus granulosus* sensu lato genotypes G6/G7 and G8/G10 can be regarded as two distinct species. *Parasitology*. © Cambridge University Press.
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II. **Laurimäe, T.,** Kinkar, L., Romig, T., Omer, R.A., Casulli, A., Umhang, G., Gasser, R., Jabbar, A., Sharbatkori, M., Mirhendi, H., Ponce-Gordo, F., Lazzarini, L., Soriano, S.V., Varcasia, A., Rostami-Nejad, M., Andresiuk, V., Maravilla, P., Gonzalez, L., Dybicz, M., Gawor, J., Šarkunas, M., Snabel, V., Kuzmina, T., Saarma, U. (2018). The benefits of analysing complete mitochondrial genomes: deep insights into the phylogeny and population structure of *Echinococcus granulosus* sensu lato genotypes G6 and G7. *Infection, Genetics and Evolution.* © Elsevier.
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III. Kinkar, L., **Laurimäe, T.,** Sharbatkhori, M., Mirhendi, H., Kia, E.B., Ponce-Gordo, F., Andresiuk, V., Simsek, S., Lavikainen, A., Irshadullah, M., Umhang, G., Oudni-M'rad, M., Acosta-Jamett, G., Rehbein, S., Saarma, U. (2017). New mitogenome and nuclear evidence resolves the phylogeny and taxonomy of the highly zoonotic tapeworm *Echinococcus granulosus* sensu stricto. *Infection, Genetics and Evolution*, 52, 52–58. © Elsevier.
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IV. **Laurimäe, T.,** Kinkar, L., Andresiuk, V., Haag, K.L., Ponce-Gordo, F., Acosta-Jamett, G., Garate, T., Gonzalez, L.M., Saarma, U. (2016). Genetic diversity and phylogeography of highly zoonotic *Echinococcus granulosus* genotype G1 in the Americas (Argentina, Brazil, Chile and Mexico) based on 8279 bp of mtDNA. *Infection, Genetics and Evolution*, 45, 290–296. © Elsevier. doi: https://doi.org/10.1016/j.meegid.2016.09.015

Personal contribution of the author to the publications referred to in this thesis is as follows:

I, II and **IV** – participation in laboratory genetic analyses, data analyses and in writing of the manuscript

III – participation in laboratory genetic analyses and data analyses, provided feedback on the manuscript

1. INTRODUCTION

Tapeworms belonging to the species complex of *Echinococcus granulosus* sensu lato (s. l.) are the cause of a zoonotic disease known as cystic echinococcosis (CE). As this disease is highly prevalent in numerous regions of the world, including the Mediterranean area, Middle East, and parts of South America (Eckert et al. 2001; WHO, 2017), the impact of CE on livestock and human health is of considerable importance. The annual economic costs due to treatment of human cystic echinococcosis and losses to the livestock industry are estimated to be around 2–3 billion euros (WHO, 2017).

The general life cycle of the species complex of *E. granulosus* s. l. involves various canids as definitive hosts, whereas domesticated and wild large mammalian herbivores act as intermediate hosts (Moks et al. 2006; Schurer et al. 2014; Laurimaa et al. 2015; Thompson, 2017). The definitive host harbours the adult worm, which is located in the small intestine of the carnivorous animal. The infection in the defnitive host is generally asymptomatic since the adult stage of this parasite is a small flatworm that is only 2–7 mm in length. However, the larval stage in the intermediate host is in the form of fluid-filled cysts, which typically develop in the liver and/or in the lungs (Eckert et al. 2001; Thompson, 2017). Among other factors, the severity of the disease in the intermediate host largely depends on the location of the cyst in the internal organ, as well as on the size of the developing cyst. Humans are considered as accidental dead-end intermediate hosts for this parasite (Alvarez Rojas et al. 2014).

1.1. Mitochondrial DNA and genotypes

It has long been established that the genetic diversity of the parasite species complex of *E. granulosus* s.l. is considerably high, and on the basis of mitochondrial DNA it was initally divided into genotypes named G1-G10 (Bowles et al. 1992, 1994; Scott et al. 1997; Lavikainen et al. 2003). Genotype G9 is now, however, considered as invalid and thought to be a microvariant of genotype G7 (Thompson, 2008), and the validity of genotype G2 has remained ambiguous as well. Over the years the evidence has been accumulating that several of these recognised genotypes exhibit differences in their lifecycles, host ranges and morphology (Thompson and McManus, 2002; Romig et al. 2017; Thompson, 2017). Therefore it has been suggested to grant a number of these genotypes species status: G1-G3 as *E. granulosus* sensu stricto (s.s.), G4 as *E. equinus* and G5 as *E. ortleppi* (Thompson and McManus, 2002; Lymbery, 2017). The species status of genotypes G6-G8 and G10 has, however, remained a controversial subject, and the evidence to regard G1-G3 as one species has also been inconclusive.

The intermediate host ranges of the *E. granulosus* s.l. species complex vary immensely. Genotypes G6 and G7 are typically found to be infecting domestic ungulates as intermediate hosts, while dogs act as the final hosts for both of these genotypes. The intermediate hosts for genotype G6 are mainly camels (Asia, Africa, Middle East) and goats (South America), whereas for genotype G7 the usual intermediate hosts are domestic pigs (Cardona and Carmena, 2013; Romig et al. 2017). Nonetheless, it has also been reported that there exists some overlap in the lifecycles of G6 and G7 – genotype G7 has been recorded in goats and G6 in pigs (e.g. Varcasia et al. 2007; Aaty et al. 2012; Addy et al. 2012). There is also evidence of both G6 and G7 infections from sheep, cattle and humans (e.g., Harandi et al. 2002; Turcekova et al. 2003; Pednekar et al. 2009; Ibrahim et al. 2011; Casulli et al. 2012; Hajialilo et al. 2012; Umhang et al. 2014). In fact, genotype G6 is the second most common genotype to infect humans worldwide, and G7 infections in humans are also more frequent than previously thought (e.g. Jabbar et al. 2011; Dybicz et al. 2013; Alvarez Rojas et al. 2014). Contrary to the genotypes G6 and G7, which are typically associated with domestic animals, genotypes G8 and G10 are mainly circulating in a sylvatic cycle. Wild cervids such as moose and reindeer act as the common intermediate hosts for both G8 and G10, whereas wolves are utilised as final hosts.

Out of all the genotypes in the *E. granulosus* s.l. complex, genotype G1 has the widest host range, with sheep acting as the main intermediate host. Infections with this genotype are also frequently reported from cattle, goats, and pigs, but there have also been reports from alpacas and kangaroos, for example (e.g. Kamenetzky et al. 2002; Haag et al. 2004; Sanchez et al. 2012; Andresiuk et al. 2013; Cardona and Carmena, 2013). Infections of the dubious genotype G2 have been recorded from sheep, and in some instances also from cattle (e.g. Haag et al. 2004; Pednekar et al. 2009). Genotype G3 is generally associated with buffaloes, but sheep, cattle and camels are also suspectible to infections with G3 (e.g. Maillard et al. 2009; de la Rue et al. 2011; Sharbatkhori et al. 2011). Moreover, genotypes G1-G3 are all known to be infective to humans as well, and around 88% of human cystic echinococcosis infections worldwide have been associated with these genotypes, the majority of them with G1 (Alvarez Rojas et al. 2014).

1.2. Distribution ranges

The distribution ranges of the genotypes G6-G10, which still have a controversial species status, are predominantly allopatric. Genotypes G6 and G7 are more with a southern distribution area, with G6 being more prevalent in camel-raising areas of Africa and Middle East, and G7 having a more extensive range in pig-rearing regions (Mediterranean area, Central America) (e.g., Varcasia et al. 2006, 2007; Cardona and Carmena, 2013; Umhang et al. 2014; Lymbery et al. 2015). Both G6 and G7 have, however, also been reported to co-occur in Turkey, Argentina and Peru (e.g., Moro et al. 2009; Šnabel et al. 2009; Soriano et al. 2010; Simsek et al. 2011). Compared to G6 and G7, genotypes G8 and

G10 have been found to have a more northern range, with a sympatric distribution in the northern hemisphere – mostly in northern parts of Europe (e.g. Estonia, Finland, Sweden and Latvia), Northern Asia and Canada (e.g. Moks et al. 2006, 2008; Konyaev et al. 2013; Schurer et al. 2014; Marcinkute et al. 2015; Oksanen and Lavikainen, 2015).

Genotypes G1-G3 are generally thought to have a largely sympatric distribution, with G1 considered as the most widespread genotype worldwide (Cardona and Carmena, 2013). It has been identified from numerous countries in Africa, Eurasia, South America, North America and Australia. Cystic echinococcosis (CE) is a significant public health concern especially in South America, as it is considered to be endemic or even hyperendemic. A large proportion of these CE infections in livestock and in humans are in fact caused by genotype G1 (e.g. Kamenetzky et al. 2002; Andresiuk et al. 2013). These endemic areas in South America include regions of Southern Brazil, Argentina, Chile, Uruguay and Peru – areas where animal husbandry is widespread and frequently the main source of income (McManus et al. 2003).

1.3. Taxonomy

The taxonomy of *E. granulosus* s. l. has been a challenging issue for decades. As briefly mentioned before, while the species status of several of these genotypes seems to have been resolved, the species status of genotypes G6-G8, G10, as well as of G1-G3 remain to be clarified. The phylogeny and species status of G6-G10 has been a particularly controversial topic of debate (Moks et al. 2008; Thompson, 2008; Saarma et al. 2009; Knapp et al. 2011, 2015; Lymbery et al. 2015; Yanagida et al. 2017).

Previous studies have mainly been based on the mitochondrial DNA (mtDNA) when trying to resolve the phylogeny and taxonomic status of genotypes G6-G8 and G10, and of G1-G3 (e.g. Moks et al. 2008, Nakao et al. 2013). These studies have shown that the two genotypes (G8 and G10) involved in the sylvatic cycle are not monophyletic in the mtDNA phylogeny, but instead G10 is placed as a sister taxon to the camel/pig genotypes G6/G7. As a result it was suggested to combine G6-G10 into a single species – *E. canadensis* (Nakao et al. 2007; Hüttner et al. 2008). What is more, the mtDNA phylogeny demonstrated that the *E. granulosus* complex is paraphyletic, since mtDNA data placed *E. multilocularis* amid the *E. granulosus* s.l. genotypes (reviewed in Knapp et al. 2015). However, it is important to acknowledge that while mtDNA sequences are useful and widely used markers, they represent the evolutionary history of the maternal lineage and the maternal lineage can have a different evolutionary trajectory than that of the species. This can happen once a new mtDNA mutation becomes fixed in a population and the new mtDNA lineage diverges from the ancestral one. Mutations continue to fix independently in both the ancestral and the new mtDNA lineage (Saarma et al. 2009). However, this does not necessarily mean that these now separate lineages have become separate biological species

with little or even no genetic exchange happening between them. Genetic exchange between taxa can only be assessed with markers from the nuclear genome.

So far only two studies have used nuclear markers in order to clarify the taxonomy of the genus *Echinococcus*. Using sequences of five nuclear genes, Saarma et al. (2009) showed that the "cervid" genotypes G8 and G10 are placed as sister taxa in nuclear phylogeny, contradicting the previous mtDNA phylogeny. Additionally, according to nuclear genes, *E. multilocularis* was placed clearly separate from the *E. granulosus* s.l. complex. However, no clear distinction was made in this study between G6 and G7, and therefore the exact phylogenetic relations of all genotypes in the G6-G10 complex remained ambiguous. On the other hand, another study based on a different set of nuclear markers demonstrated that similarly to the mtDNA phylogeny, the *E. granulosus* s.l. complex could be paraphyletic (Knapp et al. 2011). Unfortunately this study did not include the genotype G10, and as a result the exact phylogenetic relations in the G6-G10 group remained unresolved. Consequently, no consensus has been reached so far. It has been suggested to treat G6-G10 as a single species (e.g. Nakao et al. 2007; Moks et al. 2008; Romig et al. 2015), or as two (G6/G7 and G8/G10; Thompson, 2008; Saarma et al. 2009), or even three (G6/G7; G8; G10) species (Lymbery et al. 2015). A recent study based on analysis of two nuclear genes suggested some nuclear allele sharing between genotypic groups G6/G7 and G8/G10 in a region of supposed sympatry in eastern Russia (Yanagida et al. 2017), supporting the single-species view. Furthermore, while crucially necessary, nuclear evidence to explicitly confirm the divison of G1-G3 into a single species is lacking – no study involving nuclear markers has previously specifically included all three of these genotypes.

1.4. Phylogeography and genetic diversity

From the public health point of view it is crucial to understand the transmission dynamics and both the regional and local differences in the distribution and genetic variation of different genotypes in the *E. granulosus* s.l. complex. This type of data is especially important for genotypes responsible for the majority of both human and livestock cystic echinococcosis – namely G1, G6 and G7.

So far most of the studies that have focused on exploring the genetic diversity, phylogeographic patterns and population structures of *E. granulosus* s.l. genotypes G6, G7 and G1 have been based on relatively short sequences of mtDNA – generally a gene fragment, but lately also on a single gene. While shorter sequences might be cost efficient and to some extent useful tools, the analytical power for these types of studies has remained somewhat limited. The evolutionary networks have typically had a "star-like" configuration since most of the samples have clustered into a small number of central haplotypes (e.g. Haag et al. 2004; Casulli et al. 2012; Yanagida et al. 2012; Andresiuk et al. 2013; Addy et al. 2017). Consequently, determining the actual mtDNA variability, evolutionary

relations and potential phylogeographic patterns has therefore been challenging. Furthermore, even the distinction between the two closely related genotypes G6 and G7 has in some cases been ambiguous as a number of samples have not corresponded to either of the genotype clusters and have instead positioned between G6 and G7. Consequently, assigning them to either of the genotypes has remained questionable based on short sequences of mtDNA (e.g. Nakao et al. 2013; Addy et al. 2017). On the other hand, analysis of complete mitochondrial genomes or near-complete mitogenomes instead of short mtDNA sequences has shown great promise in phylogeographic analyses for other taxa in the animal kingdom. For example, based on complete mitogenome sequences of brown bears, population structuring and phylogeographic processes were revealed (Keis et al. 2013; Anijalg et al. 2018) that had not been detected previously using shorter sequences (Davison et al. 2011).

1.5. Aims of the thesis

The main objectives of the present thesis were:

1) to resolve the taxonomic status of the controversial *E. granulosus* s.l. genotypes G6-G10. In order to achieve this goal, six nuclear loci were sequenced for samples of G6, G7, G8 and G10 in paper **I**;
2) to analyse the genetic diversity and population structure of genotypes G6 and G7. For this purpose, complete mitochondrial genomes were sequenced for the first time for a relatively large number of G6 and G7 samples collected from various countries worldwide in paper **II**;
3) to reveal the taxonomic status of *E. granulosus* s.s. genotypes G1-G3. In paper **III** near-complete mtDNA sequence data was used to correctly assign samples to genotypes, and three nuclear genes were analysed to help confirm the taxonomic status;
4) to elucidate the phylogeographic patterns and genetic variability of *E. granulosus* s.s. genotype G1 collected from several endemic regions of South and North America by sequencing near-complete mitochondrial genomes in paper **IV.**

2. MATERIAL AND METHODS

2.1. Parasite material

Altogether 188 *E. granulosus* s.l. samples were analysed. These samples originated from various regions of the world (4 continents, 26 countries), and were collected from a total of ten different intermediate, as well as from one final host species. Out of the overall sample size (n=188), 60 isolates were identified as *E. granulosus* s.s. genotype G1, and 13 as genotype G3, one isolate as *E. equinus* (G4), three as *E. ortleppi* (G5), 27 as *E. granulosus* s.l. genotype G6 and 67 as genotype G7, whereas eight samples corresponded to genotype G8 and nine to genotype G10. Samples were ethanol-preserved at –20 °C until further use.

The intermediate host species genotype G1 samples were collected from were cattle (n=32), sheep (n=24), human (n=1), buffalo (n=1), and pigs (n=2). Genotype G3 samples originated from sheep (n=8), buffalo (n=1), camel (n=3) and cattle (n=1). The one genotype G4 sample was obtained from a donkey. For the three samples of genotype G5, the intermediate host species was the buffalo, while the intermediate host species genotype G6 samples were obtained from were goats (n=6), camels (n=8), cattle (n=3), sheep (n=6) and humans (n=4). Genotype G7 samples were from pigs (n=62), sheep (n=2), as well as from humans (n=3). Isolates of genotype G8 were collected from the intermediate host species of moose (n=3), and from the final host wolf (n=5). Genotype G10 samples were acquired from moose (n=5) and reindeer (n=4).

2.1.1. Parasite material for taxonomy of G6-G10

The total number of samples of *E. granulosus* genotypes G5-G10 analysed for the taxonomy study (**I**) based on six nuclear genes was 41 (see Fig. 1 and Table 1 in **I**). The two genotype G5 samples included in this study originated from India. Samples of genotype G6 (n=8) were from Argentina (n=3), Kenya (n=1), Sudan (n=2) and Iran (n=2). Genotype G7 isolates (n=14) were from Argentina (n=3), Spain (n=1), France (n=7), Romania (n=1) and Italy (n=2). Samples of genotype G8 (n=8) originated Estonia (n=3) and Latvia (n=5), while genotype G10 (n=9) samples were from Sweden (n=1), Finland (n=4), Russia (n=3), and Estonia (n=1).

2.1.2. Parasite material for phylogeny and genetic diversity of G6 and G7

In paper **II**, a total of 94 samples of *E. granulosus* s.l. genotypes G6 and G7 were included in the phylogenetic and population structure analyses based on complete mitochondrial genomes (see Fig. 1 and Table S1 in **II**). This also

included the 22 samples of G6 (n=8) and G7 (n=14) for which we previously sequenced nuclear genes for in paper **I**.

Genotype G6 samples (n=27) were collected from Sudan (n=14), Kenya (n=3), Mauritania (n=2), Iran (n=4), Mongolia (n=1) and Argentina (n=3). Genotype G7 samples (n=67) originated from Spain (n=1), France (n=27), Italy (n=2), Serbia (n=1), Romania (n=2), Ukraine (n=2), Poland (n=5), Lithuania (n=1), Mexico (n=10) and Argentina (n=16).

2.1.3. Parasite material for taxonomy of G1-G3

A total of 27 samples of *E. granulosus* s.s. (G1-G3; n=23), *E. equinus* (G4; n=1) and *E. ortleppi* (G5; n=3) were included in the analyses in paper **III** based on three nuclear genes and mitochondrial sequences (see Fig.1; Table 1 in **III**). The samples of *E. granulosus* s.s. originated from 10 different countries: India (n=2), Iran (n=4), Turkey (n=4), Spain (n=5), France (n=3), Finland (n=1; human sample, patient was from Algeria), Chile (n=1), Argentina (n=1), Albania (n=1) and Tunisia (n=1). The one *E. equinus* (G4) sample originated from Turkey, and the three *E. ortleppi* (G5) samples were collected from India. The mtDNA sequences for samples from Chile (n=1) and Argentina (n=1) were also included in the analyses in paper **IV**. Additionally, two of the *E. ortleppi* (G5) isolates were used in analyses in **I**.

2.1.4. Parasite material for phylogeography of G1 in the Americas

E. granulosus s.s. genotype G1 samples (n=52), which were included in the phylogeography and genetic diversity study based on mitochondrial DNA in paper **IV**, originated from four countries in the Americas: Argentina (n=36), Brazil (n=9), Chile (n=6) and Mexico (n=1) (see Fig. 1 and Table 1 in **IV**).

2.2. DNA extraction, PCR and sequencing

For all four papers (**I, II, III, IV**), the isolation of genomic DNA from cyst membranes, protoscoleces or adult worms was done using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocols.

PCR reactions in papers **I**, **II, III** and **IV** were carried out in a total volume of 20 µl, using 1 × BD Advantage 2 PCR buffer (BD Biosciences, Franklin Lakes, NJ, USA), 0.2 mM dNTP (Fermentas, Vilnius, Lithuania), 0.25 µM of each primer, 1 U Advantage 2 Polymerase mix (BD Biosciences) and 20–50 ng of purified genomic DNA.

2.2.1. Sequencing six nuclear loci for resolving the taxonomy of G6-G10

Six nuclear genes were chosen for PCR amplification and sequencing: transforming growth factor beta receptor kinase (*tgf*; 1137 bp), calreticulin (*cal*; 1138 bp), elongation factor 1 alpha (*ef1*; 1055 bp), ezrin-radixin-moesin-like protein (*elp*; 780 bp), phosphoenolpyruvate carboxykinase (*pepck*; 1506 bp), and DNA polymerase delta (*pold*; 1771 bp). For further details on cycle parameters and primer pairs for PCR and sequencing see Saarma et al. (2009; *tgf, cal, ef1, elp*) and Knapp et al. (2011; *pepck, pold*). For accurate genotype identification, complete mitochondrial genomes were also sequenced for the 41 samples which were analysed, and a median joining network was calculated (for further details on genotype identification see section 2.3, and for complete mitogenome sequencing see **II**). Nuclear sequences were deposited in GenBank (accession numbers: MG766944–MG767169).

2.2.2. Phylogeny and genetic diversity of G6-G7 – complete mitogenome sequencing

In order to obtain complete mitochondrial genomes for paper **II**, PCR amplification was carried out using altogether 13 primer pairs. Out of these 13, two primer pairs were identical to those also described in paper **III**, and ten were as also described in paper **IV**, whereas one primer pair was newly designed (see Table 1 in **II**). PCR and sequencing conditions were as described in paper **IV**. All complete mitochondrial genome sequences were deposited in GenBank (accession numbers: MH300929–MH301022; see Table S1 in paper **II**).

2.2.3. Sequencing of mtDNA and three nuclear loci for taxonomy of G1-G3

A total of 12 primer pairs were used in order to obtain near complete mitochondrial DNA sequences for *E. granulosus* s.s. G1-G3 samples (see Table 2 in **III**). For additional details on cycle parameters refer to paper **IV**. All mtDNA sequences were deposited in GenBank (KY766882-KY766908).

Three nuclear genes were also chosen for sequencing, with the total sequence length of 2984 bp. These three nuclear loci (*tgf*, 937 bp; *cal*, 1272 bp; *ef1*, 775 bp) were also included in the analyses in paper **I,** but with different lengths. For further details on cycle parameters and primer pairs for PCR and sequencing refer to Saarma et al. (2009). All nuclear genes were deposited in GenBank (KY766909–KY766920).

2.2.4. mtDNA sequencing for phylogeography of G1 in the Americas

In order to analyse a large portion of the mitochondrial DNA of the 52 samples of genotype G1, originating from endemic areas of South and North America, a total of 10 primer pairs were used (see Table 2 in **IV**). For further details on PCR conditions, PCR product purification and sequencing refer to paper **IV**. All mtDNA sequences were deposited in GenBank (accession numbers KX039937-KX039965).

2.3. Sequence assembly and mtDNA genotype identification

Consensus sequences for papers **I**, **II**, **III** and **IV** were assembled using Codon Code Aligner software v5.0.1 or v.6.0.2. BioEdit v.7.2.5 software was used for Clustal W multiple sequence alignment, and to manually check and correct the sequences for errors (Thompson et al. 1994; Hall, 1999). In order to confirm genotype designations, mitochondrial genome sequences from GenBank were compared with the mitochondrial sequences obtained for papers **I**, **II**, **III** and **IV**. Reference mtDNA sequences for genotype assignment were as follows: genotype G1 NC008075 (Yang et al. 2005), G3 KJ559023 (Wang et al. 2016), G4 AB786665 (Nakao et al. 2013), G5 AB235846, G6 AB208063, G7 AB235847, G8 AB235848 (Nakao et al. 2007), and for genotype G10 AB745463 (Nakao et al. 2013).

2.4. Data analysis

2.4.1. Evolutionary network and Bayesian phylogeny of G6-G10

In order to visualise and confirm genotypes and their distances, the program Network v.4.612 was used to draw the median joining network based on complete mtDNA sequences of G5-G10 samples (n=41) which were analysed in paper **I**.

Bayesian phylogenies were constructed for two datasets, both based on six nuclear genes (7387 bp in total): 1) Dataset 1 (a total of 40 sequences): 39 samples of G6-G8 and G10 analysed in this study, and one additional G8 sample from GenBank, originating from the USA (accession numbers for pepck and pold were FN567995 and FN568366, respectively; Knapp et al. 2011); 2) Dataset 2 (a total of 42 sequences): the same set of samples as in Dataset 1, and two additional sequences of genotype G5.

The best fit nucleotide substitution model was selected on the basis of Bayesian Information Criterion (BIC) scores using jModelTest 2 (Guindon and Gascuel, 2003; Darriba et al. 2012). Bayesian phylogenetic analysis was performed in BEAST 1.8.4 (Drummond et al. 2012) using StarBeast (Heled and

Drummond, 2010). Posterior distributions of parameters were estimated by using the Markov Chain Monte Carlo (MCMC) sampling. Total length of the chain was 10 000 000 and the parameters were logged every 1000 generations. The resulting phylogenetic trees were summarized and annotated using TreeAnnotator 1.8.4, and visualised with FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

In addition, it has previously been reported that there are a few polymorphic sites in nuclear loci in the G6-G10 genotype complex where the same mutations are shared between G6/G7 and G8/G10 genotypic groups (Yanagida et al. 2017). Therefore we also checked our aligned nuclear sequences for polymorphic sites that were able to differentiate between genotypes, as well as for positions where the same mutation was shared between G6/G7 and G8/G10 genotypic groups.

2.4.2. Phylogeny and genetic diversity of G6-G7 – evolutionary network, Bayesian phylogeny and population indices

Two sample sets used for network calculations in paper **II** were as follows: dataset A (n=94, complete mitochondrial genome sequences, 13 552–13 556 bp); dataset B (n=94, *cox1* gene sequences, 1608 bp). For details on network calculations see paper **III** or section 2.4.3 in the current thesis.

The best fit nucleotide substitution model for the dataset was determined in PartitionFinder 2.1.1 (Guindon et al. 2010; Lanfear et al. 2012, 2016). Bayesian phylogeny was inferred for the dataset A (complete mitogenomes) using program BEAST v1.8.4 (Drummond et al. 2012). Markov Chain Monte Carlo (MCMC) sampling was used to assess the posterior distribution of parameters (chain length 50 000 000 states, 10% burn-in). Parameter behaviour was assessed in Tracer v1.6 (Rambaut et al. 2014). Phylogenetic trees were summarised using TreeAnnotator v.1.8.4, and the resulting tree was visualised in the program FigTree v.1.4.3 (Rambaut, 2014).

Population diversity indices for complete genome sequences (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated in DnaSP v5.10.01 (Librado and Rozas, 2009). Neutrality indices Tajima's D and Fu's Fs, and the pairwise fixation index (Fst) were estimated in Arlequin v.3.5.2.2 (Tajima, 1989; Fu, 1997; Excoffier et al. 2005). Diversity and neutrality indices were calculated separately for genotypes G6 and G7, and for the two G7 haplogroups: G7a and G7b. Fst values were calculated between two large genotype clusters of G6 and G7; as well as between G6 and the two G7 haplogroups (G7a, G7b).

2.4.3. Taxonomy of G1-G3 – phylogenetic network

Two median joining networks were constructed using the program Network v4.612 (Bandelt et al. 1999; http://www.fluxus-engineering.com, Fluxus Technology Ltd., 2004): one for the mitochondrial DNA dataset, and the other for nuclear loci. Both indels and point mutations were considered for network calculations.

2.4.4. Phylogeography of G1 in the Americas – phylogenetic network and indices

In paper **IV**, median joining networks were created as described in paper **III**. Population diversity indices (such as haplotype diversity Hd, nucleotide diversity π) and neutrality tests (Tajima's D, Fu's Fs) were calculated for total set of samples and haplogroups with more than 5 haplotypes using DnaSP v5.10.1 (Librado and Rozas, 2009).

3. RESULTS

3.1. Taxonomy of G6-G10

3.1.1. Median joining network based on complete mitochondrial genomes

The total length of complete mitochondrial sequences for 41 successfully amplified samples was 13 550–13 552 bp. The median joining network revealed that all mitochondrial genotypes (G5-G10) were clearly separate, forming distinct clusters (Fig. 1). Genotype G5 (*E. ortleppi*) was the most distant genotype from all others and it appeared that more than 1200 mutations separated G5 from G8, >1000 mutations from G6-G7 cluster, and >800 mutations from G10. However, the distance between G8 and G6-G7 was also remarkable: >600 mutations. It appeared that G10 was closer to G6-G7 cluster than to G8. Genotypes G6 and G7 were the most closely related, but nevertheless clearly separated from each other by a minimum of 28 mutations.

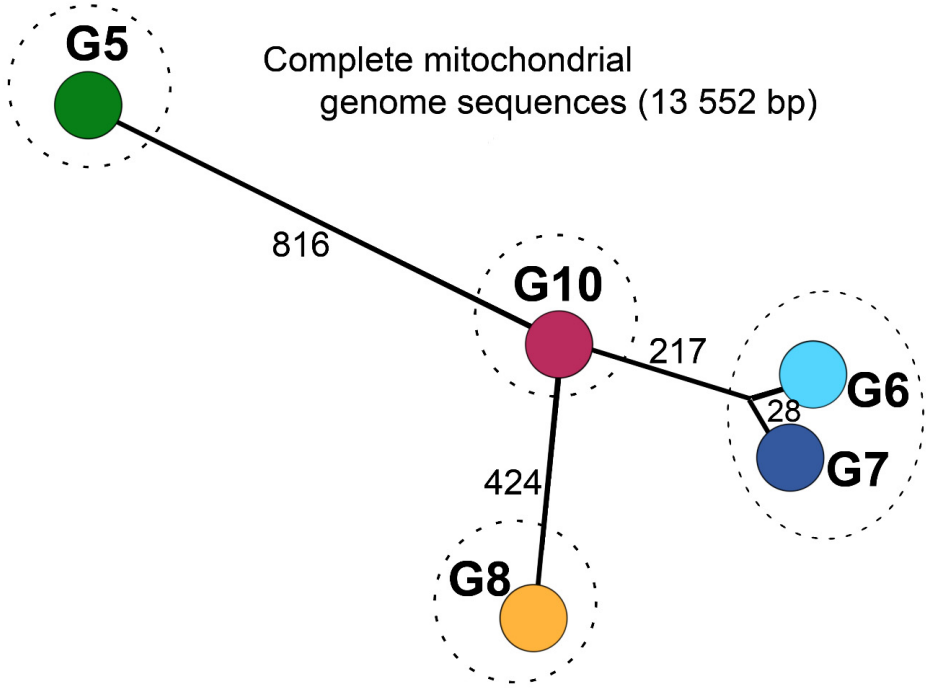

Figure 1. Schematic representation of the median joining phylogenetic network based on complete mitogenome sequences of genotype G5-G10 samples. Numbers above the lines represent the number of mutations. Green stands for genotype G5 (*E. ortleppi*), pink is for genotype G10, orange for G8, light blue for G6 and dark blue stands for genotype G7.

3.1.2. Bayesian phylogeny based on nuclear genes

Total length of the alignment based on six nuclear loci was 7387 bp: *ef1* 1055 bp, *cal* 1138 bp, *tgf* 1137 bp, *elp* 780 bp, *pepck* 1506 bp, and *pold* 1771 bp. However, a few of the samples did not yield positive results for all analysed nuclear loci, but as BEAST allows analysis with some missing data, these samples were also included in the analysis (see Table 1 in **I**). All of the samples were homozygotes at all six nuclear loci.

The best fit nucleotide substitution model used for the nuclear DNA (nDNA) data was GTR+I+G. The Bayesian phylogeny for the Dataset 1 revealed that genotypes G6 and G7 formed one clade, whereas G8 and G10 another (Fig. 2 in **I**). Posterior probability values for both nodes assigning G6/G7 and G8/G10 into two different clades were very high (1.00). According to the evolutionary (general lineage) species concept they can be regarded as two distinct species.

Internal nodes for the clade G8/G10 also received high posterior probability values (0.98 and 1.00). It was shown that G8b (the GenBank sample from USA) was a sister taxon to G10d (Estonia), and that G10c was a sister taxon to the G8b/G10d clade. Similarly to G8/G10 clade, the internal nodes for G6/G7 also received high posterior probability values (0.96 and 1.00). The resultant tree topology shows that G6 is a sister taxon to G7e, and that G7d is sister to G6/G7e. G7c occupied a basal position inside the G6/G7 clade.

We also performed a phylogenetic analysis for the Dataset 2 (included G5), as well as with only the samples for which all six nuclear loci were sequenced (Table 1 in **I**). These analyses yielded essentially the same phylogenetic relations between G6-G10 as with the larger dataset (Fig. 2; Fig. S1, S2, S3 in **I**).

In addition, across the six nuclear loci there were 12 polymorphic positions that discriminated between G6/G7 and G8/G10, whereas in the *pepck* locus mutations in three positions were shared between two G7 isolates (samples 13 and 14) and G8/G10 isolates. According to GenBank reference FN567995 (Knapp et al. 2011) these positions were: 236; 1435–1436; 1513.

3.2. Genetic diversity and phylogeny of G6 and G7

3.2.1. Phylogenetic network based on full mitogenome sequences

The length of the complete mitochondrial genome sequences of dataset A varied between 13 552 bp and 13 556 bp. According to the final designation of genotypes based on complete mithochondrial sequences, 67 of the 94 samples analysed in this study corresponded to the genotype G7-cluster, and 26 samples to the genotype G6-cluster (Fig. 2 in **II**). One sample (Gmon) positioned clearly separate from both of the genotype clusters, with 33 mutations separating Gmon from the G6-cluster, and 31 mutations from the G7-cluster.

Genotype G6 was represented by 15 different haplotypes which formed a single coherent cluster (Fig. 2 in **II**). Samples which were collected from

relatively close geographical areas were generally also genetically close; e.g., most of the samples from Sudan represented one major haplotype (SUD1), whereas SUD2 and SUD3 positioned 5–6 mutations apart from SUD1. However, several samples that were obtained from geographically distant locations were genetically close to one another; e.g., samples from Iran (IRA3) and Mauritania (MAU1) were separated by 2 mutations, and ARG3 from Argentina was separated from both IRA3 and MAU1 by 4 mutations.

Genotype G7 was divided into 44 different haplotypes, which formed two distinct haplogroups G7a and G7b. G7a and G7b represented 37 and 7 haplotypes, respectively. Usually, samples from geographically close locations were positioned together in the network (Fig. 2 in **II**). Specifically, samples from Argentina (ARG4-ARG11) formed one small haplotype cluster within haplogroup G7a, separated by 1 to 3 mutations. A similar situation was seen with the haplotypes from Corsica island (FRA1-FRA8) that formed a small monophyletic cluster within haplogroup G7a, with a maximum of 17 mutations separating the most distant haplotypes (FRA2, FRA3 from FRA7, FRA8).

Although most G7a samples from close locations were genetically similar, some samples from similar locations were genetically distant from one another. For example, two haplotypes from Mexico MEX9 and MEX6 were separated by 22 mutations, and ARG12 was separated from other Argentinian samples (ARG4-ARG11) by a minimum of 23 mutations. Furthermore, ARG12 was more closely related to haplotypes from geographically distant locations, namely Lithuania (LIT1) and Poland (POL1-POL3).

The smaller haplogroup G7b included 12 samples originating from Corsica island (France, n=10) and Sardinia island (Italy, n=2). Haplogroup G7b was distant from G7a, separated by 20 mutations. It is important to note that according to the originally published G6 and G7 *cox1* sequences (366 bp) by Bowles et al. (1992), samples representing G7b would have been classified as genotype G6. However, the whole mitochondrial genome sequences positioned these samples on the median joining network within the G7 cluster.

3.2.2. Bayesian phylogeny

The best fit model determined in PartitionFinder for the dataset A was GTR+I+G. The resultant Bayesian phylogeny inferred from complete mitochondrial genome sequences divided the 94 samples into three large, well supported clades: G6, G7a and G7b (Fig. 3 in **II**). Posterior probability (PP) values for all of these clades were maximum (PP=1.00). The highly divergent sample (Gmon) from Mongolia assumed a sister clade position in relation to the G6 cluster (PP=0.94).

There was no clear substructuring within the G6 clade according to geographic location. However, the internal division of G7a displays two highly supported monophyletic clades (PP=1.00) with samples from France (FRA1-FRA8) and from Argentina (ARG4-ARG11) each clustering together. Similarly, G7b samples originating from Corsica island (France) and Sardinia island

(Italy) clustered together (PP=1.00). The remainder of samples from close locations did not form location-specific clades.

3.2.3. Population neutrality and diversity indices

The haplotype diversity value Hd for genotype G7 was 0.967, and nucleotide diversity π was 0.00150. For genotype G6, the respective Hd value was 0.828, and π was 0.00040 (Table 2 in **II**). Haplogroup G7a was characterised by high Hd value (0.959), while for G7b it was somewhat lower (0.833). For haplogroup G7a π was 0.00122, and for G7b this value was marginally lower (0.00026).

Genotype G6 had a statistically highly significant Tajima's D value of –1.780, whereas for genotype G7 only Fu's Fs was statistically significant (Table 2 in **II**). For haplogroups, significant values were attributed to haplogroup G7a, whereas for G7b, the neutrality indices were not significant.

3.2.4. Population differentiation

The pairwise fixation index between genotypes G6 and G7 was high (Fst=0.680) and statistically highly significant ($p< 0.00001$; Table S2 in **II**). The Fst value between the divergent Gmon and genotype G6 was higher (0.846, $p<0.05$), while somewhat lower (0.564, $p<0.01$) between Gmon and G7.

Fst values for haplogroups were statistically highly significant between G6 and G7a (0.746, $p < 0.001$), between G6 and G7b (0.891, $p< 0.001$) and between G7a and G7b (0.604, $p< 0.001$) (Table S3 in **II**). However, the Fst values between Gmon and G7 haplogroup G7a (0.669), as well as between Gmon and G7b (0.916) were high, but not statistically significant.

3.2.5. Phylogenetic networks based on *cox1*

We compared the phylogenetic power of complete mitogenomic datasets versus the *cox1* gene only (the most frequently used marker in *Echinococcus* studies). To do this, we constructed separately the networks using the complete mitogenome (13 552–13 556 bp) and the *cox1* (1608 bp) datasets.

Based on the mitogenome network, genotypes G6 and G7 represented two distinct and well-supported clusters, with substructuring within G7 (haplotypes G7a and G7b). By contrast, using only the complete *cox1* gene there was no unequivocal distinction between genotypes G6 and G7 (Fig. 4 in **II**). Moreover, the highly divergent Gmon sample (mitogenome network) was not discernable in the *cox1* network, as it grouped together with 11 other samples in the central haplotype (hap1) of G6.

3.3. Taxonomy of G1-G3

A total of 27 samples (G1, n=10; G3, n=13; G4, n=1; G5, n=3) were successfully sequenced, with final mtDNA sequence lengths varying between 11 442–11 466 bp. Whereas all three nuclear loci (*cal, ef1, tgf*) were also successfully amplified for the same set of samples. The only sample that did not yield a positive PCR product with nuclear markers was one putative genotype G2 sample from Spain (SPA3).

3.3.1. mtDNA phylogenetic network

The mtDNA network based on nearly complete mtDNA sequences revealed that *E. granulosus* s.s. (G1-G3) samples were separated from *E. equinus* (G4) samples by 1244, and from *E. ortleppi* (G5) samples by 1387 mutations. The number of mutations between *E. equinus* and *E. ortleppi* was 1228 (Fig. 2 in **III**).

Samples of *E. granulosus* s.s. (G1-G3) were divided into two larger haplogroups with the genetic distance between them being 37 mutations (Fig. 2 in **III**). The mtDNA sequences of the haplogroup comprising ten isolates were highly homologous with the available sequences of genotype G1 in the GenBank database (reference sequence AF297617; Le et al. 2002) and was therefore named accordingly, whereas the second haplogroup with 13 isolates corresponded to genotype G3 (GenBank reference KJ559023; Wang et al. 2016).

Out of the ten haplotypes in the G1 cluster, six contained the original genotype G1 *cox1* (366 bp) sequence fragment described by Bowles et al. (1992). These haplotypes were as follows: FIN1, TUN1, ARG1, TUR4, CHI1 and IND2. In the genotype G3 haplogroup, seven haplotypes out of 13 included the originally described *cox1* sequence fragment of genotype G3 by Bowles et al. (1992) (SPA1, SPA2, SPA4, IRA1, IRA2, IRA3, FRA2). However, three of the samples (SPA3, TUR2, TUR3), which comprised the original genotype G2 *cox1* sequence by Bowles et al. 1992, did not form a separate haplogroup, but instead clustered inside the G3 haplogroup. Furthermore, these putative G2 samples were not positioned monophyletically inside the G3 cluster.

3.3.2. nDNA phylogenetic network

According to three nuclear genes (*ef1*, *tgf* and *cal*) the 26 samples that were successfully sequenced in paper **III** were divided on the phylogenetic network into three distinct clusters – *E. equinus* (G4), *E. ortleppi* (G5) and *E. granulosus* s.s. (G1/G3) (Fig. 3 in **III**). Based on the nDNA phylogenetic network, the genetic distance between *E. granulosus* s.s. (G1/G3) samples and *E. ortleppi* (G5) was 36 mutations, whereas between *E. granulosus* s.s. (G1/G3) and *E. equinus* (G4) it was 45 mutations. *E. equinus* (G4) was separated from *E. ortleppi* (G5) by 23 mutations.

3.4. Genetic diversity and phylogeography of G1 in the Americas

Final length of mtDNA sequences for the analysed *E. granulosus* s.s. genotype G1 samples (n=52), which originated from South and North America, was 8279 bp. This included 15 full length coding regions and 6 gene fragments (Table 3 in **IV**).

3.4.1. Phylogenetic network

The median joining network revealed that the 52 samples of genotype G1 were divided altogether into 29 separate haplotypes and clustered into five distinct haplogroups, named H1 to H5 (Fig. 2 in **IV**). The number of haplotypes in each of the haplogroups were as follows: haplogroup H1 comprised 15 haplotypes (n=32 isolates) with one central haplotype ARG1, H2 included one haplotype (n=2 isolates); H3 two (n=2 isolates), whereas haplogroup H4 contained eight haplotypes (n=12 isolates) with distant lineages and no central haplotype, and haplogroup H5 consisted of three haplotypes (n=4 isolates).

Samples originating from regions in close proximity to each other, were also genetically more closely related. For example, in haplogroup H1 most of the samples from Argentina's Buenos Aires province (n=16) clustered into one central dominant haplotype ARG1, whereas the rest of the haplotypes in H1 (e.g. ARG2-ARG10) were positioned around ARG1. Similarly in haplogroup H5, haplotypes from geographically close locations from Chile were positioned only one mutation apart (CHI1 and CHI4).

Additionally, a number of samples from geographically distant locations were genetically more closely related to each other than to those originating from the same region. For instance, in haplogroup H4 isolates from Brazil (e.g. BRA3) were separated from isolates obtained from Argentina (e.g. ARG12) only by one mutation, whereas other isolates from Brazil formed haplotypes BRA1 and BRA6 that clustered into a separate haplogorup H1, and were positioned eight mutations apart from BRA3. These haplotypes BRA1 and BRA6 from Brazil were instead more closely related to the central haplotype ARG1 in haplogroup H1. Similar situation was also seen in haplogroup H2 and H3 where samples from Argentina and Brazil were genetically more similar to each other than to other isolates from the same localities (e.g. BRA2 and ARG11 in H3).

The phylogenetic network, however, also revealed as expected that samples from distant locations were genetically more distant. E.g. haplotype described from Mexico (MEX1) was placed 14 mutations apart from a haplotype obtained from Argentina's Buenos Aires province (ARG14), and 12 mutations apart from a haplotype described from the southern region of Brazil (BRA4).

3.4.2. Population indices

Total haplotype diversity (Hd) index for the 52 analysed samples was 0.90, with a nucleotide diversity (π) of 0.00077 (Table 4 in **IV**). Neutrality indices were also calculated for the total set of samples: Tajima's D was –2.20, with a statistically significant p-value (<0.01), and Fu's Fs –12.60.

4. DISCUSSION

4.1. Taxonomy of G6-G10

A stable taxonomy of *E. granulosus* s.l. is essential to the medical and veterinary communities for accurate and effective communication of the role of different species in this species complex on both human and animal health. However, the taxonomic status of *E. granulosus* s.l. genotypes G6-G10 has still remained a controversial issue (Saarma et al. 2009; Knapp et al. 2011; Lymbery et al. 2015; Nakao et al. 2015). So far, the majority of studies aiming to resolve the taxonomy of these genotypes have been based on mitochondrial DNA (e.g. Lavikainen et al. 2003; Nakao et al. 2007; Hüttner et al. 2008; Moks et al. 2008; Nakao et al. 2013). Nonetheless, it is essential to acknowledge that mtDNA reveals only the evolutionary history of the maternal lineage which can differ from that of the whole species. Moreover, for accurate species delimitation the possibility of genetic exchangeability between different genotypic groups is one of the key components that needs to be analysed. This can only be studied by using nuclear markers (Saarma et al. 2009). Until recently, only two studies have relied on nuclear loci for *E. granulosus* s.l. phylogeny analyses, with contradictory results (Saarma et al. 2009; Knapp et al. 2011). Unfortunately neither of the studies explicitly included all four of the genotypes in the G6-G10 complex.

The results of our study for complete mitogenome sequencing of genotypes G5-G10 clearly demonstrated and confirmed the distinct nature of each of the genotypes included in the analyses for paper **I** (Fig. 1 in the current thesis). As expected, the most distant genotype from all others was G5 (*E. ortleppi*), and the most closely related genotypes were G6 and G7. However, while G6 and G7 were shown to be genetically closely related according to mtDNA, they still formed clearly distinct genotype clusters and can therefore be firmly regarded as separate mitochondrial genotypes.

As the Bayesian phylogeny, which was based on six nuclear loci, showed the division of the camel/pig genotypes G6/G7 into one clade, and the wild cervid genoytpes G8/G10 into another, we concluded that this result provides strong support for the hypothesis according to which the G6-G10 genotype complex is divided into two separate species (Fig. 2 in **I;** Thompson, 2008; Saarma et al. 2009). However, while our nuclear data suggested limited or even non-existent gene flow between G6/G7 and G8/G10, the internal divison of the G6/G7 clade suggests there exists sufficient gene flow between G6 and G7 to guarantee that the two genotypes have not diverged from each other (notice in Fig. 2 in paper **I** that G7e formed a subclade with G6, while other isolates of G7 were sister to this). Similar situation could be seen with the G8/G10 clade – G8 and G10 did not form separate subclades, the isolates of both genotypes were not monophyletic.

The possibility for gene flow between genotypic groups can only occur when these genotypes have a sympatric distribution – to date none of the studies have

demonstrated sympatry of all of these four genotypes. So far there are only a limited number of recorded instances where potential sympatry of G6/G7 and G8/G10 genotypic groups has been identified. One such region is in Far East Russia, where G6 has been found from close proximity (~500 km) to the G8 and G10 genotypes (Konyaev et al. 2013). Another region for potential sympatry for these genotypic groups is in north-Eastern Europe, where G8 (**I**) and G10 have been recorded from Latvia, and G7 has been documented from Lithuania (Marcinkute et al. 2015). Considering that wolves, which are the main final hosts for G8/G10, are able to travel very long distances, and since it has also been demonstrated that the wolf populations in Europe are indeed connected over the distance of more than 800 km (Hindrikson et al. 2017), then the possibility for gene flow between G7 and G8/G10 is feasible. Nevertheless, the genotypic groups G6/G7 and G8/G10 form separate clades based on nDNA phylogeny and according to the evolutionary (general lineage) species concept these two clades can be regarded as distinct species since they represent two distinct evolutionary lineages.

However, a recent study based on two nuclear loci (*pepck* and *pold*) with samples from the potential region of sympatry in Far East Russia, suggested there is some evidence of gene flow between G6 and G8/G10 since there were a few shared alleles between these two genotypic groups (Yanagida et al. 2017). In fact, there were a number of polymorphic positions in our sequences as well (including *pepck* and *pold*), where the same nucleotide was shared between G6/G7 and G8/G10 genotypic groups. For example, in *pepck* all isolates of G8 and G10 have A in the shown position, however A is also remarkably found in two isolates of G7 (Fig. 3 in **I**). Yet there are enough characters only specific either to G6/G7 or to G8/G10 that in the phylogeny they form two separate clades. So while we cannot rule out the possibility that to some extent gene flow between the two genotypic groups might occur, it is also essential to acknowledge that limited gene flow between species, i.e. hybridisation, is in fact relatively common in nature. In general, it has been estimated that 10–30% of multicellular animal and plant species hybridise regularly (Abbott et al. 2013). Hybridisation is also well-known among parasites, it is known for example between different species of helminths (*Taenia, Trichinella, Schistosoma, Fasciola, Ascaris*) (Arnold, 2004; Detwiler and Criscione, 2010; King et al. 2015). The occurrence of hybridisation does not mean that the two hybridising species, if clearly separate on the phylogeny, should therefore be regarded as a single species, it just means that reproductive barrier between species has not yet fully developed.

What is more, the division of G6-G10 into two separate species is also supported by other relevant ecological, epidemiological and morphological data that can be found in detail in Thompson (2008) and Saarma et al. (2009). Briefly, for example while G6/G7 is known to be typically circulating in the domestic cycle (camels/goats/pigs) with dogs most likely providing opportunities for outcrossing, G8/G10 cycles primarily in the sylvatic cycle, between cervids (moose/elk/reindeer) and wolves (Thompson and McManus, 2002; Lymbery,

2017). Although there are only a few recorded instances of G6 in northern latitudes (Konyaev et al. 2013; Yanagida et al. 2017), the geographical distribution ranges are still largely allopatric for the two genotypic groups – G6/G7 are more with a southern distribution range, and G8/G10 are in contrast distributed in the northern parts of Eurasia and North America (Lavikainen et al. 2003; Thompson et al. 2006; Moks et al. 2008). Therefore the probability that parasites from different genotypic groups co-occur in the same definitive host and cross-fertilize is very low. Thus we concluded that the association with distinct host species, largely separated geographical distribution and limited rate of cross-fertilization are the main factors that have likely limited the gene flow between genotypic groups G6/G7 and G8/G10. As a result, these genotypic groups can be regarded as distinct species.

Based on priority, the species name for G8/G10 should be *E. canadensis*, while the species name for G6/G7 warrants further discussion. It has been proposed to use *E. intermedius* for G6/G7 (Thompson, 2008; Saarma et al. 2009). However, this name is highly problematic since the original description by Lopez-Neyra and Soler Planas (1943) did not include a description of the intermediate host and no original type specimen for *E. intermedius* can be found (Nakao et al. 2015).

4.2. Genetic diversity and phylogeny of G6 and G7

As we previously demonstrated in paper **I** that while G6 and G7 form one species, they do still represent two distinctive mitochondrial lineages. In paper **II** we aimed to elucidate on the genetic diversity and phylogeographic patterns of these two epidemiologically important genotypes – G6 is the second most frequent genotype to infect humans, and CE infections with genotype G7 are also more common than previously thought (Jabbar et al. 2011; Dybicz et al. 2013; Alvarez Rojas et al. 2014; Ito et al. 2014). Yet, data on the genetic diversity and phylogeography of these two genotypes is relatively scarce. The little that is known has been based on relatively short fragments of the mitochondrial *cox1* and *nad1* genes, and occasionally on full length gene sequences of these aforementioned genes (e.g. Addy et al. 2017). However, to gain deeper insight into the genetic diversity, phylogeny and phylogeography of *E. granulosus* s.l., studies using large portions of mitogenomic sequences have started to appear, providing more reliable genotypic classification, and yielding profounder insight into their large-scale phylogeographic patterns (e.g. Kinkar et al. 2016, 2018a, 2018b). In paper **II**, for the first time complete mitogenome sequences (13 552 bp – 13 556 bp) were used to analyse the genetic diversity and phylogenetic relations of G6 and G7 collected from various countries worldwide. It is important to note that complete mitogenomes provide the ultimate resolution that can be obtained for mtDNA.

G6 and G7 formed two major genotypic clusters based on the phylogenetic network inferred from complete mtDNA sequences (Fig. 2 in **II**). This was

already briefly demonstrated with a smaller sample size in **I** as well (Fig.1. in the current thesis). Additionally, the Bayesian phylogenetic tree also gave strong support for the existence of two mtDNA genotypic groups – posterior probability values for both nodes assigning the samples into two different clades was high (Fig. 3 in **II**). The calculated population differentiation value between G6 and G7 further indicated these mtDNA genotypic groups are separated (Table S2 in **II**). So far it has been occasionally problematic to correctly assign haplotypes to either G6 or G7, when relying on relatively short mtDNA sequences, with the authors having to classify them as G6/7 instead. This has been due to the fact that based on relatively short sequences, intraspecific genetic variation within G7 has often been higher than between G6 and G7, making the assignment of samples that positioned between both genotypes challenging (e.g. Nakao et al. 2013; Addy et al. 2017). However, accurate genotype assignment is essential, particularly from an epidemiological point of view.

Interestingly, one sample from a human patient from Mongolia (Gmon) was genetically distinct from both G6 and G7 (Fig. 2 in **II**). However, in the Bayesian phylogeny Gmon positioned as a sister group to the G6 clade (Fig. 3 in **II**), suggesting a closer evolutionary relationship with G6 than with G7. However, high Fst values between Gmon and both genotypes suggests that this haplotype is very different from both G6 and G7 (Table S2 in **II**). Further analysis of additional samples from Mongolia and other remote regions could help establish whether there are more divergent lineages of *E. granulosus* s.l. Although highly divergent mitochondrial haplotypes, such as Gmon are rare, another was recently found in Ethiopia and tentatively classified as belonging to *E. granulosus* s. s., awaiting further confirmation (Wassermann et al. 2016).

Genotype G6 formed a coherent cluster with no clear segregation of samples according to geographic location (Fig. 1 and Fig. 2 in **II**). The high haplotype (Hd = 0.828) and low nucleotide diversity indices (π=0.0004), together with the significantly negative Tajima's D (D=–1.780, $p \leq 0.01$) suggest rapid population expansion following the recurrent introduction of the parasite into different regions through animal trade.

Contrary to the single coherent cluster of genotype G6, the network analysis of genotype G7 revealed two different haplogroups G7a and G7b (Fig. 2 in **II**). This divison into G7a and G7b received further support from the Bayesian phylogeny since the posterior probability values for the nodes assigning G7a and G7b into separate clades were maximum (PP=1.00; Fig. 3 in **II**). Morever, the population differentiation analysis also gave strong support for the existence of two haplogroups (Fst=0.604, $p<0.001$; Table S3 in **II**). Intriguingly, G7b represents samples exclusively from the neighbouring islands of Corsica (France) and Sardinia island (Italy). A similar genetic structuring of samples from Corsica island (France) was recently found based on shorter sequences (*cox1* and *nad1*), although it remained unclear to which of the genotypes these samples belonged, since they were placed in an intermediate position between genotypes G6 and G7 (Umhang et al. 2014; Addy et al. 2017). The authors proposed that it is possible that this type of genetic structuring is due to the

restricted gene flow between these islands and surrounding mainland areas, as it is hypothesised that the intermediate hosts for this genotype (pigs) were introduced into the area during the Neolithic period and have remained in relative isolation ever since (Larson et al. 2005; Albarella et al. 2006; Addy et al. 2017). It is also possible that the domestication of livestock animals has given grounds to an accelerated adaption and genetic diversification of parasites to their respective local livestock host species (Badaraco et al. 2008).

We also compared the resolution of phylogenetic networks inferred from complete mitogenome data (13 552–13 556 bp) and *cox1* (1608 bp) gene. This comparison demonstrated that limited conclusions can be drawn from analyses using short sequences. This is highly important in light of the fact that even the distinction between genotypes G6 and G7 has in some cases been unclear based on *cox1*. The *cox1* gene phylogenetic network of the current study exhibited a "star-like" structure. The highly divergent Gmon haplotype and haplogroup G7b were only detectable with complete mitogenome data. Therefore we concluded that complete mtDNA sequence data is particularly useful in phylogeographic and population structure analyses for it allows us to achieve the maximum resolution and to gain a deeper insight into the phylogeny of the parasite.

4.3. Taxonomy of G1-G3

In paper **III**, we sequenced nearly complete mitogenomes and three nuclear genes in order to confirm and provide evidence for the species status of *E. granulosus* s.s. G1-G3. So far, sample genetic characterization as genotypes G1-G3 has frequently been without a distinct definition. Numerous samples do not explictly correspond to the original description of genotypes in Bowles et al. (1992). Moreover, similarly to genotypes G6 and G7 (**I** and **II**), it has sometimes been difficult to clearly allocate some of the samples into genotypes G1–G3 due to the lower discriminatory power of short mtDNA sequences, which was also discussed in paper **II** (e.g. Romig et al. 2015; Wassermann et al. 2016). Whereas the near complete mtDNA sequences of paper **III**, however, allowed for a clear differentiation of genotypes since samples of G1-G3 formed two distinct mtDNA haplogroups corresponding to mitochondrial genotypes G1 and G3 (Fig. 2 in **III**). The three putative samples of genotype G2, which had identical *cox1* (366 bp) sequences of the molecular definition originally published by Bowles et al. (1992), clustered together with G3 samples. Moreover, as they did not form a monophyletic cluster inside the G3 haplogroup, we therefore concluded that genotype G2 is not a valid mitochondrial genotype and should be excluded from the list.

As it was also argued in papers **I** and **II** for genotypes G6/G7 and G8/G10, since our nuclear data shows no differentiation between genotypes G1/G3 (Fig. 3 in **III**), the fact that mitochondrial data shows clearly separate genotypes does not mean that these genotypes have become separate biological entities. So, whilst our nuclear data of three loci was able to distinguish between geno-

types G4 (*E. equinus*) and G5 (*E. ortleppi*) and G1/G3 (*E. granulosus* s.s.), there was no distinction of G1 and G3 separate clusters. Consequently, since G1 and G3 only have limited ecological differences with a largely sympatric distribution and frequently also share same or similar definitive and intermediate hosts, we concluded that G1 and G3 can be regarded as a single species (*E. granulosus* s.s.). The results of paper **III** are the first to provide evidence for this, and as our results were unequivocal, we would also argue that the number of samples analysed was sufficient to verify the species status of *E. granulosus* s.s. Had the results unexpectedly suggested that G1 and G3 formed two different species, then there would have been a need for including a larger number of samples.

4.4. Genetic diversity and phylogeography of G1 in the Americas

Previously, in paper **III** we firmly demonstrated the distinctive nature of *E. granulosus* s.s. genotype G1 in the context of mtDNA. In paper **IV** genetic diversity and phylogeographic patterns of G1 in endemic regions of South and North America were studied. The intraspecific phylogeographic patterns of genotype G1 have largely remained unresolved since the resultant phylogenetic networks have mostly yielded star-like networks due to the common practice of using only one gene, or often one gene fragment (e.g. Haag et al. 2004; Badaraco et al., 2008; Nakao et al. 2010; Sanchez et al. 2010; Casulli et al. 2012; Andresiuk et al. 2013; Sharma et al. 2013). However, as we already demonstrated earlier, longer sequences enable us to obtain a much higher resolution (**II**), and in paper **IV** we also attempted to improve the phylogenetic resolution for *E. granulosus* s.s. genotype G1 by analysing sequences of a large portion of the mitogenome, covering 15 different mitochondrial loci and 6 gene fragments (8279 bp in total; which is about 60% of the mitochondrial genome).

The observed overall haplotype diversity of *E. granulosus* s.s. genotype G1 in South and North America was relatively high (Hd=0.90) with 52 of the samples dividing into 29 haplotypes that clustered into 5 haplogroups (H1-H5) (Fig. 2 and Table 4 in **IV**). The high haplotype diversity was somewhat expected since for G1 it has previously been hypothesised to be attributed, among other factors, to the wide host range (low host specificity), which in turn has helped faciliate the global distribution of G1 (e.g. Kamenetzky et al. 2002; Casulli et al. 2012). Moreover, the existence of different genetic variants is also partly due to the relatively high mutation rate of the mitochondrial DNA, the short generation time of the parasite and the fact that the larval stage has a high proliferation rate and high prevalence of G1 in America (Thompson and McManus, 2002; Haag et al. 2004; Saarma et al. 2009).

Additionally, no distinct phylogeographic segregation pattern of samples nor haplogroups according to geographic location could be identified. Samples

collected from remote locations were frequently genetically closely related (e.g. BRA2 and ARG11; CHI2 and ARG13), whereas isolates from neighbouring localities were observed to be genetically distant from each other (e.g. ARG8 and ARG14; Fig. 2 in **IV**). Similarly to the results of paper **II**, such lack of geographic segregation suggests the importance of animal transportation in shaping the current population structure of *E. granulosus* s.s. G1. Indeed, in both Argentina and Brazil animal husbandry is widespread and transportation of animals between countries has been historically frequent. However, the likely origin of G1 is hypothesised to be in the Middle East (Eckert et al. 2001; Kinkar et al. 2016, 2018) and the introduction of the genotype G1 through domestic animals into the Americas has historically likely been a recurrent event.

The most abundant haplotype (ARG1) in haplogroup H1 could be due to a relatively large number of samples analysed from the Buenos Aires region in Argentina and should therefore not be regarded as the most prevalent haplotype in America. Its central position, however, suggests that it could be the ancestral haplotype for H1. Interestingly in paper **II** the G7 samples from Argentina also showed similar clustering of samples around one central haplotype (Fig. 2 in **II**). The existance of a single central haplotype, together with the lower haplotype and nucleotide diversities of samples from Argentina (G7: Hd=0.733, π=0.00061; Table 2 in **II;** G1: 0.805, π=0.0005 in **IV**), could be explained by the relatively recent introduction of the parasite into the area through trade of its intermediate or final hosts.

4.5. Conclusions

The results of this thesis clearly demonstrated that the controversial genotype complex of G6-G10 is in fact divided into two separate species based on six nuclear genes – G6/G7 and G8/G10 (**I**). Nevertheless, based on mitochondrial genomes they do represent distinct mitochondrial lineages. Therefore we also looked at the genetic diversity and population structure of G6 and G7 based on complete mitogenomes (13 552– 13 556 bp) as from the public health point of view they represent an important topic of interest (**II**). Since there was no distinctive segregation pattern of samples according to location, we proposed that animal transportation has had a significant impact on the population structure of G6 and G7. On the other hand, for G7 there were a number samples that formed clusters according to location, and thus geographic isolation has also played a role. Moreover, G7 was represented by two haplogroups G7a and G7b, and one significantly divergent sample was identified from Mongolia (Gmon). However, these distinctions of G7a, G7b and Gmon could not be made with the commonly used *cox1* (1608 bp) gene sequences. Therefore we proposed that to gain a better and deeper understanding of actual genetic diversity and population structures, longer sequences of mtDNA are of great benefit.

We also provided conclusive evidence to regard G1/G3 as one species *E. granulosus* s.s. according to nuclear genes, and as separate genotypes only in

the context of mtDNA (**III**). The results of mitochondrial sequences also suggested that genotype G2 is not a valid mitochondrial genotype and we suggested to exclude genotype G2 from the genotype list. Furthermore, as G1 is the most frequent genotype to be associated with human infections, we also analysed the genetic diversity and phylogeography of G1 in areas of South and North America where G1 is highly prevalent (**IV**). The analysis based on mtDNA sequences showed high genetic diversity and no clear segregation pattern according to geographic location, indicating similarly to G6 and G7 that animal tranpsortation has likely had a strong influence on the population structure of G1 as well.

In conclusion, the current thesis significantly helped improve our knowledge on the taxonomy, genetic diversity and phylogeography of the tapeworm species complex of *E. granulosus* s.l.

SUMMARY

Tapeworms of the species complex of *Echinococcus granulosus* sensu lato (s.l.) are the cause of a severe zoonotic disease – cystic echinococcosis (CE). The disease is listed among the most severe parasitic diseases in humans and is prioritised by the World Health Organisation. A stable taxonomy of *E. granulosus* s.l. is essential to the medical and veterinary communities for accurate and effective communication of the role of different species in this complex on human and animal health. This parasite complex displays high genetic diversity and has been divided into different species and genotypes. Despite several decades of research, the taxonomy of *E. granulosus* s.l. has remained controversial. While the species status of a number of genotypes has remained undisputed, the species status of genotypes G6-G10 has remained a topic of debate. No unanimous decision has been reached – some have suggested to treat them as one species, while others as two or even three species. What is more, while genotypes G1-G3 are officially regarded as one species, so far there has been no study to provide conclusive evidence to regard them as such, since no published study has unequivocally included all three genotypes G1, G2, G3 in a nuclear loci analysis. However, if we are to delimitate any species we would need to evaluate possible genetic exchangeability between all genotypes in question, which can only be done by analysis of nuclear data.

In the current thesis we aimed to resolve the taxonomic status of genotypes G6-G10 by including all four of these genotypes for the first time in a nuclear analysis of six loci (*ef1*, *tgf, cal, elp, pepck, pold*). The Bayesian phylogeny analysis firmly divided these genotypes with maximum posterior probability values (1.00) into two separate clades – the domestic camel/pig genotypes G6/G7 formed one clade, and the wild cervid genotypes G8/G10 another. This result is also supported by other ecologically relevant data, such as the predominantly allopatric distribution of G6/G7 (southern) and G8/G10 (northern) clusters, and the fact that the life cycles have major differences – G6/G7 is perpetuated in a cycle involving domestic animals (pigs, goats, camels, dogs), while G8/G10 are disseminated in a cycle between wild cervids (moose, elk, reindeer, wolves). Therefore, as our results suggested non-existent or very limited gene flow between G6/G7 and G8/G10, we concluded that according to the evolutionary species concept they can be regarded as two distinct species.

Similarly to G6-G10 this thesis also aimed to provide conclusive evidence for regarding genotypes G1-G3 as one species by sequencing three nuclear loci (*ef1, tgf, cal*). Additionally, we also sequenced near complete mitogenomes for the same set of samples to firmly designate the samples into correct genotypes, since based on short sequences of mtDNA it has occasionally been difficult to distinguish between genotypes G1-G3. The results of the mtDNA phylogenetic network analysis demonstrated that: 1) genotype G2 is not a valid genotype as the putative G2 samples clustered within the G3 haplogroup, whereas they did not form a monophyletic cluster inside the G3 haplogroup; 2) genotypes G1 and

G3 formed two clearly distinct genotype clusters, with 37 mutations between them. However, since nuclear data showed no distinction of genotype G1 and G3 clusters, we concluded that G1 and G3 can be regarded as a single species (*E. granulosus* s.s.) and that they are separate genotypes only in the context of mitochondrial data.

Furthermore, another objective of the current thesis was to elucidate and improve on the phylogeography, population structure and genetic diversity of the three most common genotypes (G1, G6, G7) to infect humans in the *E. granulosus* s.l. species complex. Due to the common practice of analysing short sequences of mtDNA, such data has often remained ambiguous since the resulting phylogenetic networks have typically been with a star-like configuration with most of the samples clustering into one or two central haplotypes. This has made it somewhat difficult to determine actual genetic diversity and population structures. Therefore we sequenced complete mitogenomes for genotypes G6 and G7 samples originating from various countries worldwide, and more than half of the mitogenome for genotype G1 samples collected from the most prevalent endemic areas in South and North America. Our results revealed that: complete or significantly longer mitogenomic sequences allow significantly better phylogenetic and –geographic resolution compared with the commonly used *cox1* gene (**II** and **IV**). Although the genotypes G6 and G7 represent two different mitochondrial lineages (**I**), the situation is more complex than previously thought (**II**): 1) the use of mitogenomic data discovered a highly divergent haplotype (Gmon) from Mongolia, which remained undetected using full *cox1* data; 2) mitogenome data revealed that genotype G7 is divided into two major haplogroups G7a and G7b, which also remained undetected when we only looked at the full *cox1* sequence data; 3) the complex genetic structure of genotypes G6 and G7 is likely associated with the trade of livestock animals, but geographic isolation has also had an influence. Likewise to genotypes G6 and G7, the mitochondrial genotype G1 (**III**) demonstrated a lack of a clear geographic segeration in the endemic regions of South and North America (**IV**). The results of **II** and **IV** strongly highlight the importance of animal transportation in shaping the phylogeographic patterns and population structure of *E. granulosus* s.l. species complex.

The current thesis considerably improved our knowledge on the taxonomy, genetic diversity and phylogeography of the zoonotic tapeworm species complex of *E. granulosus* s.l., and is an important milestone towards a better understanding of the diversity, spread and epidemiological ecology of this species complex.

SUMMARY IN ESTONIAN

Zoonootiliste paelusside liikidekompleksi *Echinococcus granulosus* sensu lato taksonoomia ja geneetiline mitmekesisus

Ehhinokokkide perekonda kuuluv põistang-paeluss (*Echinococcus granulosus* sensu lato; s.l.) on liikide kompleks, mille hulka kuuluvad parasiidid põhjustavad zoonootilist haigust nimega tsüstiline ehhinokokkoos. Maailma Terviseorganisatsioon (rahvusvaheline lühend WHO) on klassifitseerinud antud haiguse kõige raskemakujulisemate parasitooside hulka. Kuna põistang-paelussi poolt põhjustatud majanduslikud kahjud ulatuvad ülemaailmselt miljarditesse eurodesse, siis on WHO prioriseerinud tsüstilise ehhinokokkoosi ja selle haigustekitajast parasiidi laiemat uurimist (WHO, 2017).

Põistang-paelussi üldine elutsükkel eeldab kahe peremehe ja kiskja-saakloom tsükli olemasolu. Lõpp-peremeesteks on koerlased (peamiselt koerad ja hundid) ning vaheperemeesteks on sageli herbivooridest sõralised (Moks et al. 2006; Schurer et al. 2014; Laurimaa et al. 2015; Thompson, 2017). Lõpp-peremehes areneb parasiidist täiskasvanud uss, kes on keskmiselt 2–7 mm pikk ning ei põhjusta seega lõpp-peremehele suuremaid vaevuseid. Seevastu vaheperemehes hakkab arenema põistang-paelussi vastse arengustaadium, milleks on vedelikuga täidetud tsüst, kus arenevad nakkusvõimelised protoskooleksid (Eckert et al. 2001; Thompson, 2017). Vedelikuga täidetud tsüstid paiknevad vaheperemehe organismis reeglina kopsus või maksas, kuid muuhulgas on neid tuvastatud ka ajust, põrnast ja teistest organitest (Eckert et al. 2001; Thompson, 2017). Inimesed on põistang-paelussile juhuslikud n-ö tupik-peremehed (Alvarez Rojas et al. 2014). Sageli esineb nakatunud vaheperemehes, sh inimeses, üheaegselt mitu tsüsti, mis tekitavad tõsiseid terviseprobleeme ja õigeaegse ravi puudumise korral võivad põhjustada surma.

Teadaolevalt on põistang-paelussil märkimisväärselt lai geneetiline varieeruvus. Algselt tuvastati lühikeste mitokondri *cox1* (366 ap) geeni järjestuste põhjal 10 genotüüpi, mis nummerdati G1-G10 (Bowles et al. 1992, 1994; Scott et al. 1997; Lavikainen et al. 2003). Hiljem selgus, et genotüüp G9 on genotüübi G7 mikrovariant ning seetõttu on G9 nüüdseks genotüüpide nimekirjast välja arvatud. Samuti on kahtluse alla seatud genotüüp G2 kui eraldiseisev genotüüp. Ametlikult kehtivate genotüüpide vahel esineb erinevusi nende patogeensuses, vaheperemeeste eelistustes, elutsüklites ja levikupiirkondades (Thompson and McManus, 2002; Romig et al. 2017; Thompson, 2017). Eelnimetatud erinevused on andnud piisavalt alust, et klassifitseerida mõningad genotüübid eraldi liikideks. Nii otsustati geneetiliselt omavahel lähedalt seotud G1-G3 nimetada liigiks *E. granulosus* sensu stricto (s.s.), G4 on tuntud kui *E. equinus* ja G5 kui *E. ortleppi*. Seejuures on aga genotüüpide G6-G8 ja G10 liigiline staatus jäänud siiani selgusetuks. Mõned teadlased on soovitanud neid käsitleda ühe liigina (Nakao et al. 2007), teised kahena (Thompson, 2008; Saarma et al. 2009) ja kolmandad on pakkunud, et esineb kolm liiki (Lymbery et al. 2015).

Erimeelsused G6-G10 taksonoomilises staatuses on suuresti tingitud asjaolust, et suurem osa senini avaldatud fülogeneesi alastest töödest on keskendunud peamiselt mitokondri uurimisele. Mitokondri puhul on seejuures oluline meeles pidada, et see näitab ainult emaliini evolutsiooni. Liikide eristamiseks on esmatähtis koguda aga infot mõlema liini evolutsiooni kohta, kuid seda on võimalik teostada ainult tuumageene analüüsides. Tuumageene on siiani käsitlenud kaks tööd, millest kumbki ei kaasanud analüüsidesse kahjuks kõiki nelja vaidluse all olevat genotüüpi (G6, G7, G8 ja G10). Lisaks olid ka mõlema teadustöö tulemused üksteisega vastuolus (Saarma et al. 2009; Knapp et al. 2011). Probleemseks on jäänud ka genotüüpide G1-G3 käsitlemine ühe liigina, kuna polnud avaldatud ühtegi teadusuuringut, mis oleks nende liigilist kuuluvust kinnitanud tuumageenide põhjal ja seejuures veel kaasanud fülogeneesi korraga kõik kolm genotüüpi (G1, G2, G3). Stabiilne taksonoomia on samas oluline nii teaduslikust seisukohast kui ka veterinaaridele ja arstidele, et omavaheline kommunikatsioon oleks üheselt mõistetav.

Lisaks on ka inimtervise seisukohalt haiguste levikuteede ja nende võimalike regionaalsete mustrite erinevuste mõistmine olulise tähtsusega. Senised uuringud antud valdkonnas on eelkõige baseerunud lühikestel mitokondriaalsetel järjestustel, sageli põhinedes ainult ühel geenil või isegi üksikul geenifragmendil. Tulemuseks on olnud n-ö tähekujuline võrgustik, kus suurem osa proove klasterdub ühte või kahte tsentraalsesse haplotüüpi. Selle tagajärjel on senini olnud kohati keeruline kindlaks teha reaalset geneetilist varieeruvust ja levikumustreid. Nende kindlakstegemine on aga eriti oluline just kõige laiemalt levinud ja kõige sagedamini inimnakkustega seostatud genotüüpidel G1, G6 ja G7.

Käesoleva doktoritöö raames oli üheks eesmärgiks üritada lahendada põistangpaelussi genotüüpide G6-G10 taksonoomiline ja liigiline staatus. Selleks kaasasime analüüsi esmakordselt kõik neli genotüüpi (G6, G7, G8 ja G10) ning sekveneerisime kõigile neljale genotüübile kuus tuumageeni. Tuumageenide põhjal läbi viidud Bayesi fülogeneetiline analüüs näitas, et need neli genotüüpi jagunevad kahte klaadi – peamiselt koduloomadega (siga, kaamel, kits, koer) seostatud genotüübid G6/G7 kuuluvad ühte ning valdavalt metsikute sõralistega (põder, metskits, põhjapõdrad) seotud G8/G10 kuuluvad teise klaadi (**I**). Mõlema klaadi toetused olid maksimaalsed (1.00). Meie tulemust, et G6/G7 ja G8/G10 esindavad kahte erinevat klaadi, toetavad ka mitmed G6/G7 ja G8/G10 gruppide vahelised ökoloogilised erinevused. Nimelt on G6/G7 ja G8/G10 peamiselt allopatrilise levikuga: G6/G7 on laiema levikuga lõunapoolsetel aladel (näiteks Vahemeremaad, Lõuna-Ameerika, Lähis-Ida, Aafrika), kuid genotüüpide G8/G10 peamine areaal asub põhjapoolsetel aladel (näiteks Põhja-Euroopa riigid, Kanada, Venemaa põhjaosa jne). Erinevusi esineb ka elutsüklites – G6/G7 on eelkõige seostatud koduloomadega ning G8/G10 ringlevad metsloomade tsüklis. Seega leidsime, et G6-G10 genotüüpide kompleksi võiks jagada kaheks liigiks.

Sarnaselt eelpool käsitletud genotüüpidega üritasime lahendada ka genotüüpide G1-G3 liigilise kuuluvuse küsimust. Selleks sekveneerisime kõigil kolme genotüüpi esindavatel proovidel kolm tuumageeni. Lisaks sekveneerisime genotüüpide määramiseks ka peaaegu kogu mitkondri täisgenoomi. Pikemad

mitokondriaalsed järjestused olid vajalikud, kuna varasemalt oli teada, et lühemate järjestuste põhjal on kohati keeruline eristada omavahel geneetiliselt lähedalt seotud genotüüpe G1, G2 ja G3. Mitokondri analüüsi põhjal koostatud fülogeneetiline võrgustik näitas, et: 1) genotüüp G2 ei ole eraldiseisev genotüüp, kuna G2 proovid klasterdusid G3 haplogruppi ja ei moodustanud eraldi monofüleetilist rühma; 2) G1 ja G3 on mitokondri DNA analüüsi põhjal eraldiseisvad genotüüpide grupid, mis on üksteisest eraldatud 37 mutatsiooniga (**III**). Tuumageenide analüüs seevastu ei näidanud G1 ja G3 eraldatust, vaid paigutas G1 ja G3 ühte monofüleetilisse rühma. Seega, kuna ka ökoloogiliste tunnuste poole pealt G1 ja G3 vahel olulisi erinevusi ei eksisteeri, siis järeldasime, et G1 ja G3 moodustavad ühe liigi (*E. granulosus* s.s.).

Käesoleva doktoritöö raames oli veel üheks eesmärgiks tuvastada mitokondriaalsetel genotüüpidel G1, G6 ja G7 potentsiaalsed fülogeograafilised mustrid, populatsiooni struktuurid ja reaalne geneetiline mitmekesisus. Selleks sekveneerisime genotüüpide G6 ja G7 proovidele mitokondri täisgenoomid ja genotüübi G1 proovidel enamiku mitokondri genoomist. Analüüsi tulemused näitasid selgelt, et pikkade mtDNA järjestuste kasutamisel on suur eelis, sest seeläbi on võimalik selgemini tuvastada reaalset geneetilist varieeruvust ja populatsiooni struktuuri (**II, IV**). **II** tulemused näitasid, et kuigi G6 ja G7 esindavad kahte erinevat mitkondriaalselt liini (**I**), siis populatsioonide struktuurid on oodatust keerukamad: 1) täismitkondriaalsete genoomide põhjal tuvastasime Mongooliast väga divergentse Gmon haplotüübi, mida lühemate järjestuste põhjal ei olnud võimalik kindlaks määrata; 2) genotüübil G7 esineb kaks suuremat haplogruppi G7a ja G7b, mida ei olnud samuti võimalik lühemate järjestustega tuvastada; 3) genotüüpide G6 ja G7 kompleksne geneetiline struktuur, kus ei esine selget geograafilist segregeerumist, viitab loomakaubanduse suurele mõjule. Sarnasele järeldusele viitasid ka mitokondriaalse genotüübi G1 (**III**) geneetiliste mitmekesisuse ja struktuuri analüüsid – Lõuna-Ameerikast analüüsitud proovidel puudus selge klasterdumine vastavalt kogutud proovide asukohale (**IV**).

Antud doktoritöö täiendas oluliselt teadmisi *E. granulosus* sensu lato taksonoomiast, geneetilisest mitmekesusest ja fülogeograafiast ning on oluliseks teetähiseks selle zoonootilise parasiidi liikidekompleksi mitmekesisuse, leviku ja epidemioloogilise ökoloogia mõistmiseks.

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