University of South Bohemia in České Budějovice

Faculty of Science

Phylogenetic relationships and population structure of coccidia in rodent families Muridae and Arvicolidae

Master Thesis

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Annotation:

Population structure and phylogenetic relationships were studied in coccidia parasitizing the rodent families Muridae and Arvicolidae, in 40 localities in 14 European countries. Sequences of mitochondrial gene for cytochrome c oxidase subunit I (COI) and nuclear 18S rRNA gene (SSU) were used for phylogenetic analyses and for reconstruction of evolutionary relationships among coccidian species.

Declaration [in Czech]:

Prohlašuji, že jsem svoji diplomovou práci vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Bc. Anna Mácová

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Contents

1. Introduction

1.1. Coevolutionary processes and population structure in host-parasite associations

Population structure in parasites is often coupled with the host-parasite coevolutionary processes, i.e. a parallel evolutionary development of two or more species, when both/all evolve and adapt to each other over a period of time. Among typical examples of coevolution belong the relationships between pollinators (e.g. butterflies or hummingbirds) and plants (Ehrlich and Raven, 1964; Cotton, 1998), or pathogens coevolving with their hosts.

Among the most typical and often studied coevolutionary system belongs the coevolution between parasites and their hosts (Xiao et al., 2002; Štefka et al., 2009). Each participant of this relationship exerts selective pressure on the other, so they affect each other's evolution. We recognize four principal coevolutionary events behind the congruence/incongruence of the host and parasite trees: cospeciation (simultaneous speciation of a host and its parasite), duplication (independent parasite speciation, where the parasite remains associated with the ancestral host), sorting events (disappearance or extinction of a parasite on a host lineage) and host switching (colonization of a new host) (Page and Charleston, 1998; Ronquist, 1998; Legendre et al., 2002). Succession of these events forms together a complex coevolutionary process that can be studied by variety of methods, e.g. Brook's parsimony analysis (Brooks and McLennan, 2001), component analysis (Page, 1993), or the TreeMap (Page, 1994).

While these events determine the overall degree of congruence between the host and parasite phylogenies/genealogies, the structure and population genetics of each population in given time is affected by several major evolutionary processes: natural selection (survival of some trait), genetic drift (change caused by random sampling), mutations (changes in DNA sequences) and gene flow (exchange of genes between populations). Gene flow can be restricted by reproductive isolation. An example is provided by the glacial events (Webb and Bartlein, 1992). In the Quaternary period, many species of various groups of organisms from central Europe migrated to south glacial refugia. They were isolated from each other and underwent allopatric speciation and divergence. After the glacial period, they returned back, mixed together and renewed the gene flow. These events determined the formation of current European fauna.

Due to their accessibility, rodents represent suitable model organisms for analyses of these population processes, including coevolution between the recolonizing species and their parasites. Among rodents, the genera *Apodemus* (Michaux et al., 2003, 2004) and *Microtus*, that survived glacial in central Europe and in refugia in the Caucasus or the Carpathians (Jaarola and Searle, 2002; Brunhoff et al., 2003) belong to well-studied groups from the phylogeographic point of view

(Chaline and Graf, 1988; Adkins et al., 2001; Jaarola et al., 2004; Jansa and Weksler, 2004; Buzan et al., 2008). They also served as model species in several coevolutionary studies, dealing with lice (Štefka and Hypša, 2008), or nematodes (Nieberding et al., 2004, 2005). However, the most frequent and abundant parasites of these rodent genera are coccidia of the genus *Eimeria*.

1.2. Coccidia as model organisms for molecular studies

Coccidia (Apicomplexa: Conoidasida: Eucoccidiorida) are the most numerous and diversified organisms within the phylum Apicomplexa (Chromalveolata: Alveolata) (Upton, 2000). They are commonly found in all classes of vertebrates and they were also described from several invertebrates (Pellérdy, 1974; Levine, 1988; Duszynski and Upton, 2001). This large phylum contains number of genera, some of them known as important pathogens of humans and/or animals (e.g., *Babesia*, *Theileria*, *Plasmodium*, *Toxoplasma*, or *Eimeria*). Within coccidia, the families Sarcocystidae and Eimeriidae are monophyletic, as well as the subfamilies Toxoplasmatinae and Sarcocystinae. The most speciose genus of the whole group is *Eimeria* (Perkins et al., 2000) with about 1700 species described worldwide from various hosts (http://biology.unm.edu/biology/coccidia/home.html). More than 400 species of *Eimeria* have been described in rodents (Duszynski and Upton, 2001). However, the genus *Eimeria* is clearly paraphyletic; *Cyclospora* and several *Isospora* species cluster within the *Eimeria* clade (Morrison et al., 2004) (Figure 1). It is also currently obvious that the genus *Isospora* is polyphyletic (Carreno and Barta, 1999; Franzen et al., 2000; Modrý et al., 2001) - species from birds cluster within Eimeriidae, whereas species from mammals cluster within Sarcocystidae (Carreno and Barta, 1999).

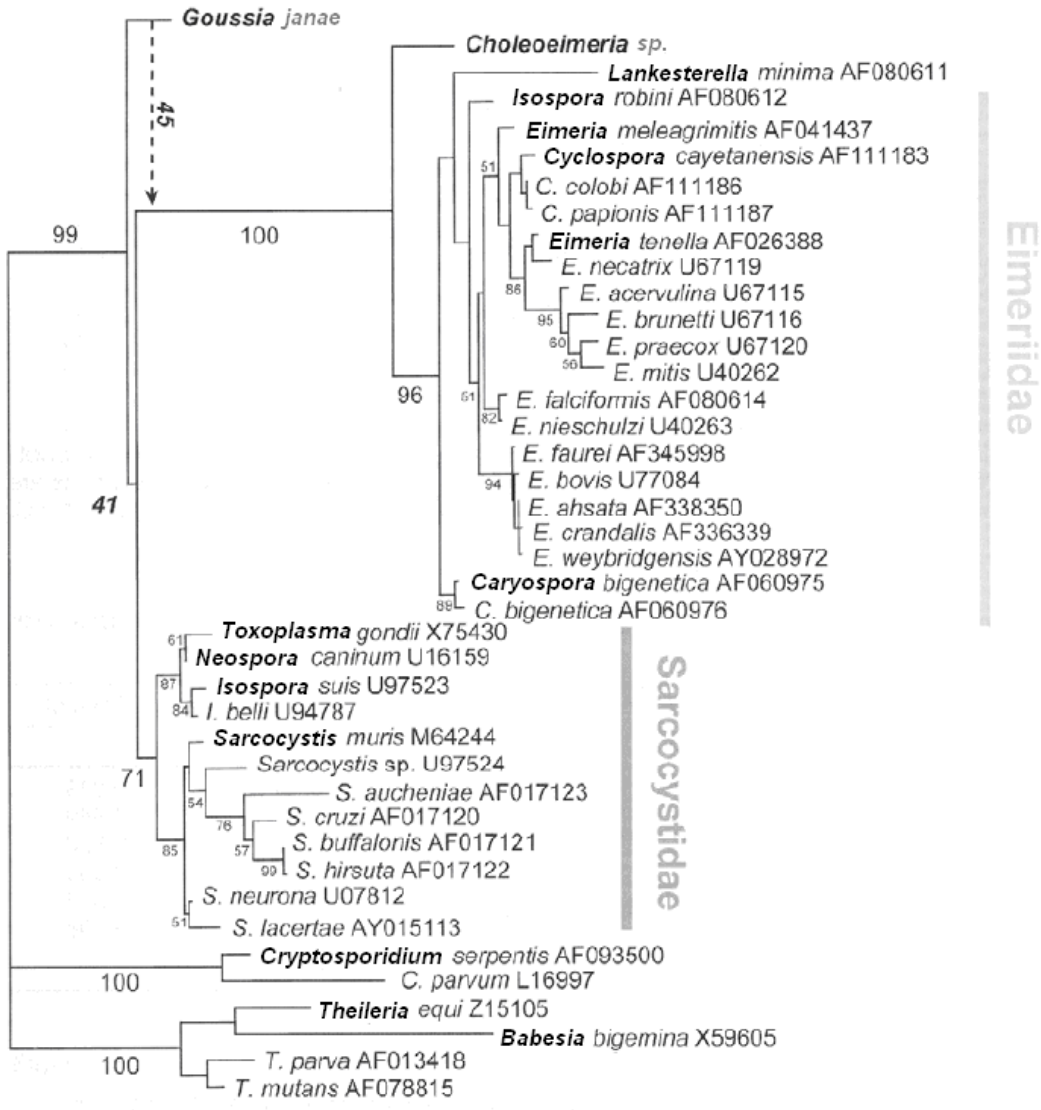

Figure 1. Phylogenetic relationships within coccidia (modified according to Jirků et al., 2002).

1.2.1. Biology of eimerian parasites

The life cycle of *Eimeria* is typically monoxenous, including both asexual and sexual reproduction (merogony and gamogony) (Kreier and Baker, 1987). They are obligate intracellular parasites of the gastrointestinal tract, with their complex life cycle located in the intestinal mucosa of the hosts. Eimerians develop in enterocytes of small or large intestine. It is remarkable that those occupying tops of duodenal or jejunal villi are less pathogenic than eimerians developing in the crypts of enterocytes of the cell lamina propria mucosae (Mesfin et al., 1978; Duszynski and Upton, 2001; Šlapeta et al., 2001). They are transmitted by the faeces of the infected animals (Berto et al., 2009). Outside the host, eimerian oocysts undergo the process of sporulation. Sporulated (i.e. infective) oocysts are highly resistant to the weather conditions. Susceptible hosts are infected *per os*, by ingestion of sporulated oocysts (Duszynski et al., 1999).

Several coccidian species are highly pathogenic for their hosts, especially in the intensive farm breedings. Coccidiosis is an important disease in poultry (Beattie et al., 2001) and rabbits (Pakandl, 2009), which can have considerable economic impact. It can be important also in other animals, causing malabsorption of nutrients and diarrhoea, due to damages in the digestive tract of their hosts (Ding et al., 2008). The degree of pathogenicity is an important trait, potentially determined by various biological characteristics of both the parasite and its host. However, despite the significance of this trait, no correlation has been found between phylogenetic relationships of coccidia and their pathogenicity (Barta et al., 1997; Kvičerová et al., 2008). On the other hand, economic impact of the pathogenic coccidia resulted in strong bias in the coccidia research: majority of the studies, and therefore majority of our knowledge, have been based on veterinary important species (Dorney, 1962; Current et al., 1990; Procunier, 1993; Barta et al., 1997).

Animals infected with *Eimeria* develop some degree of immunity. However, this immunity is not absolute and reinfections can occur (Levine and Ivens, 1965). Studies on laboratory rats revealed that stronger immunity may be caused by eimerians developing in the upper parts of the intestine, unlike the parasites from the lower parts (Becker et al., 1932; Liburd, 1973; Schito et al., 1996). The prevalence and intensity of infection can be influenced by season of the year. In Finland, Laakkonen et al. (1998) revealed that higher intensity of infection occurred in early autumn, whereas the lowest during winter, spring and early summer. This phenomenon may be due to the stronger immunity of older rodents. Several studies consider coccidiosis as a disease of young and subadult animals (e. g. Ball and Lewis, 1984), however, it was proved that it is not a rule (e.g. Stanton et al., 1992). The population of parasites in a certain host may be restricted by the host immunity. Naturally infected rodents (e. g. *Chaetodipus*, *Neotoma, Onychomys, Peromyscus,* or *Sigmodon*) were reported to contain usually a single species of *Eimeria* (McAllister et al., 1991); the same phenomenon was revealed also in *Microtus* spp. (Vance and Duszynski, 1985). However, this pattern is not present in *Apodemus* species (Higgs and Nowell, 2000). The high percentage of single infections can also signify some selective advantage for certain hosts.

1.2.2. Host specificity in eimerians

For a long time, eimerians had been supposed to be highly host-specific organisms (Hiepe and Jungmann, 1983; Rommel, 2000), and the host specificity was even considered as a suitable criterion for distinguishing the species (Joyner, 1982). However, several observations have pointed out that some species possess a broader host spectrum (Todd and Hammond, 1968a, b; de Vos, 1970; Ryff and Bergstrom, 1975; Vance and Duszynski, 1985; Duszynski, 1986; Hill and Duszynski, 1986; Upton et al., 1992; Penzhorn et al., 1994). It is probable that the occurrence in

host may be influenced also by other factors, such as geography or ecology, because some groups of mammals are more susceptible/exposed to coccidian infections due to the environment they inhabit. For example, the moist/humid environment is better for oocyst survival than xeric environment (Vance and Duszynski, 1985). The temperature and precipitation also have impact on the oocyst survival and sporulation (Dorney, 1962; Wilber et al., 1994). Cold weather provides worse conditions for sporulation (Ecke, 1956). Differences in social behaviour, life strategy and feeding habits of the hosts play an important role as well. Laakkonen et al. (1998) reported that *Microtus* (which is more sociable and searches for the food close to the ground) was more often infected by eimerians than *Clethrionomys*, which feeds higher above the ground and is less sociable.

1.2.3. Taxonomy, evolution and phylogeny of coccidian parasites

Coccidia have been described and identified mainly by morphology of sporulated oocysts (Pellérdy, 1974; Joyner, 1982; Levine, 1982; Current et al., 1990). However, such a classification may often not be exact since the range of oocyst morphology can differ within the species (De Vos, 1970; Duszynski, 1971). A remarkable inner structure of the sporulated coccidian oocyst, which presence/absence is partially congruent with the phylogeny, is the oocyst residuum (OR). It is a structure emerging during the sporulation process and is considered to be a cluster of lipid granules discarded from the cytoplasm of zygote during the sporulation. Till now, it is not known why some *Eimeria* possess this residuum, whereas others do not. The function of this structure is also unknown. It is interesting that all *Eimeria* species infecting cattle, sheep, pigs, chicken and turkeys, and more than 75 % of *Eimeria* species from snakes, were shown to lack the OR (Zhao and Duszynski, 2001b). By sequencing of the plastid ORF 470, 23S rRNA and nuclear 18S rRNA genes, it was proved that OR has a clear correlation with phylogenetic relationships (Zhao and Duszynski, 2001 a, b; Kvičerová et al., 2008). This fact supports the hypothesis about the existence of two distinct lineages of rodent *Eimeria* (Reduker et al., 1987; Hnida and Duszynski, 1999a, b; Zhao and Duszynski, 2001b). The two lineages reflect morphological differences of sporulated oocysts (absence or presence of OR) better than their host specificity. Reduker et al. (1987) hypothesized that both lineages (possessing and lacking OR) originated as sister taxa in the common ancestor of their host, or they reflect two independent events of the host invasion. According to Escalante and Ayala (1995), coccidia diverged about 800 million years ago, whereas rodents diverged less than 100 million years ago (Kumar and Hedges, 1998). This arises a question whether the common ancestor of *Eimeria* already possessed the OR, or the structure was derived during the evolution? And if the latter hypothesis is true, how old is this feature? Zhao and Duszynski (2001b) suggested that the two lineages split earlier than their hosts diverged.

Invention and usage of molecular techniques and sequencing have deeply influenced the taxonomy and systematics of coccidia. Various suitable markers and genes for analysing coccidian phylogeny were tested and studied. For instance, sequences of apicoplast genes proved more variable compared to nuclear genes, providing high amount of phylogenetically informative positions. Analysis of SSU rRNA from apicoplasts placed coccidia as a sister group to haemosporidia. Coccidia themselves split into two lineages, Eimeriidae and Sarcocystidae. Plastid sequences of coccidia evolve more slowly than in haemosporidia (Oborník et al., 2002).

Nuclear 18S rRNA (SSU) gene sequences have been often used to study phylogenetic relationships between species and/or higher taxa in Apicomplexa (Tenter and Johnson, 1997; Doležel et al., 1999; Holmdahl et al., 1999; Morrison et al., 2004). SSU rRNA gene is abundant in the genome of apicomplexans, it is a double feature of hypervariable regions in conserved DNA sequences. This gene is appropriate for inferring phylogenetic relationships in eukaryotes (Dahlgren et al., 2008). Nuclear 18S rDNA and plastid 23S rDNA proved to be good markers for reconstructing phylogenetic relationships in the genus *Eimeria*; nevertheless, they are highly conserved and therefore not suitable for resolving intraspecific variability within *Eimeria*. Similar results were obtained also by sequencing of plastid ORF 470 gene (Barta et al., 1997; Zhao and Duszynski, 2001a; Matsubayashi et al., 2005; Power et al., 2009).

Mitochondrial genes proved to be suitable markers for analysing evolutionary relationships of various organisms (e.g., Hu et al., 2002; Bellinvia, 2004; Jaarola et al., 2004; Li et al., 2008), and several coevolutionary studies were also based on this gene (e.g., Štefka and Hypša, 2008; Miska et al., 2010). Mitochondrial genes are not long, lack introns and contain short intergenic regions. Unlike the nuclear genes, they possess sufficient variability for resolving relationships on the intraspecific level (Jia et al., 2010; Miska et al., 2010). The most important fact is that mitochondria are inherited only in maternal lineage, therefore they do not undergo any radical recombinations. Till now, many sequences of complete mitochondrial genomes of Metazoa (including also *Apodemus* species) have been published (Janke et al., 1997; Hu et al., 2002; Jia et al., 2010; Oh et al., 2011; Kim and Park, 2012), but only few of them have been sequenced for parasitic protozoa (Omori et al., 2007; Hikosaka et al., 2010; Lin et al., 2011).

Other studied markers (e.g. internal transcribed spacer region – ITS, riboprinting, isoenzymes, microsatellites or random amplified polymorphic DNA) did not prove to be sufficiently effective for resolving phylogenetic relationships among/within coccidia (Bellinvia, 2004; Ogedengbe et al., 2011).

1.3. Rodents as hosts of coccidian parasites

Rodents (Mammalia: Rodentia) represent almost one half of all recent mammal species (Musser and Carleton, 1993). Monophyly of this order and relationships among its families have been often discussed. Monophyly was supported e.g. by Martignetti and Brosius (1993) or Huchon et al. (1999) based on the BC1 RNA (neutral-specific small cytoplasmic RNA) or exon 28 of the gene encoding von Willebrand Factor, whereas Janke et al. (1997) or Reyes et al. (1998) refuted it on the basis of analyses of mitochondrial genes (tRNA-Lys and 12 H-strand gene products). Recent studies suggest that Rodentia are monophyletic (Adkins et al., 2001; Huchon et al., 2002).

Muroid rodents (Rodentia: Muridae), the most diversed mammalian family, consist of more than 1300 species divided into 17 subfamilies (Martin et al., 2000; Jansa and Weksler, 2004). They represent common hosts for various parasites, such as tapeworms, roundworms, or coccidians. More than 400 species of *Eimeria* have been described from rodents (Duszynski and Upton, 2001).

1.3.1. Rodentia: Muridae: Apodemus

Field mice (*Apodemus* spp.) are members of the family Muridae, subfamily Murinae. They are dispersed in various habitats and biotopes worldwide (Jansa and Weksler, 2004). Murinae represent the largest subfamily of mammals, consisting of more than 500 species and 113 genera (Musser and Carleton, 1993).

The world is inhabited by more than 20 species of the genus *Apodemus*, half being from Europe and half from Asia (Musser and Carleton, 2005). *Apodemus* represent the most common rodents in the temperate zones in Palearctic (Orlov et al., 1996). Borders of their occurrence are not fully resolved in many localities in Europe (Hoofer et al., 2007). They inhabit various biotopes, where they find proper food, for example acorns, insect or other small invertebrates. They can be found both in natural habitats (forests, meadows) and urban areas (Montgomery and Dowie, 1993; Kaneko et al., 2008). Different species often live in sympatry, which can be associated with the biogeographic history of the genus and its speciation processes (Suzuki et al., 2008).

In history of this genus, more than one bush-like speciation burst occurred (Serizawa et al., 2000; Michaux et al., 2002; Suzuki et al., 2003), leading to origin of many new species (Orlov et al., 1996; Chelomina et al., 1998; Michaux et al., 2002; Matsubara et al., 2004). High dryness in late Miocene (7-5 mya; Fortelius et al., 2002) could cause extinction of the old European *Apodemus* lineages, with exception of ancestors of *A. mystacinus* that remained in refugias in Eastern or Central Europe (Suarez and Mein, 1998; Liu et al., 2004). The genus underwent a wide dispersion and radiation 6 mya and in Pliocene (5-2 mya; Fortelius et al., 2002), when vegetation in Europe

changed mainly into forests; regionally specific radiation in Europe and south China and westward dispersion of *A. agrarius* into Europe occurred in the late Quaternary (Suzuki et al., 2008). The main phase of speciation of the genus *Apodemus* has lasted for a long time (about 10-12 mya), from Pleistocene till now (Balakirev et al., 2007). From one of the most recent radiation event (approximately 2.2-3.5 mya; Michaux et al., 2002), the subgenus *Sylvaemus* s. str. (except *A. epimelas* and *A. mystacinus*) was arisen (Figure 2).

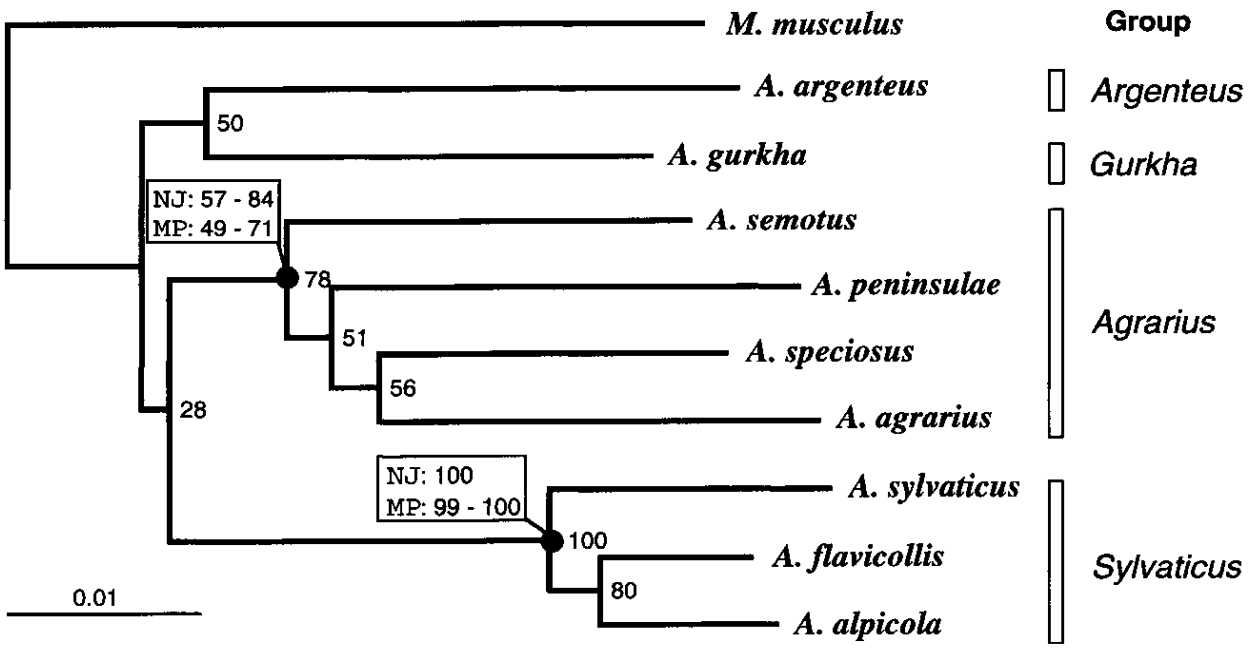

Figure 2. Phylogeny of *Apodemus* species based on cytochrome *b* gene (from Serizawa et al., 2000).

The genus *Apodemus* is divided into 3-4 subgenera. Valid taxonomy (Musser et al., 1996; Serizawa et al., 2000; Suzuki et al., 2003) distinguishes 2 main subgenera - *Apodemus* (mainly species from eastern Asia and *A. agrarius* with discontinual Eurasian range) and *Sylvaemus* (most of European and Near East species). Some authors suggest that Japanese species *A. argenteus* and Nepal endemit *A. gurkha* constitute the third and fourth discrete subgenera (Suzuki et al., 2003). *A. mystacinus* is sometimes considered as a member of *Sylvaemus* (Musser et al., 1996), but other studies place it into a distinct subgenus *Karstomys* (Michaux et al., 2002) based on morphology, chromosome and genetic data (Martin et al., 2000). The latest classifications based on molecular methods indicate 4 different lineages of the genus *Apodemus*: *Sylvaemus, Apodemus, A. argenteus* and *A. gurkha* (Musser at al., 1996; Liu et al., 2004; Suzuki et al., 2008); however, relationships among them remain still unclear (Balakirev et al., 2007).

Relationships within the genus *Apodemus* have been extensively studied. Morphology and morphometry proved to be not sufficient or accurate enough; for example, misidentification often occurred in the *Sylvaemus* group due to morphologic similarities (Miller, 1912; Đulić and Tvrtković, 1974). Therefore, more precise methods have started to be employed. Geometry of the skull or teeth morphometry belong to the current methods based on morphology (Rohlf and Marcus, 1993; Slice, 2005; Frynta et al., 2006; Barčiová, 2009). The systematics had become more reliable after employment of molecular methods based on analyses of various genetic markers (Michaux et al., 2002; Bellinvia, 2004), for example mtDNA, nuclear DNA, proteins, microsatellites, or analyses of chromosome structure (Bulatova et al., 1991; Michaux et al., 1998; Macholán et al., 2001; Chelomina and Suzuki, 2006; Balakirev et al., 2007). Most of *Apodemus* species were revealed to possess a high level of interspecific variability, but a low level of intraspecific variability (Chelomina, 1998; Serizawa et al., 2000; Liu et al., 2004; Hoofer et al., 2007). In *Apodemus flavicollis*, a surprisingly high level of haplotype diversity was observed (Michaux et al., 2004; Hoofer et al., 2007). Due to the low intraspecific variability and high interspecific differentiation, the mitochondrial genome has become a useful tool for discrimination at the species-specific level (Hoofer et al., 2007).

Two species have been of particular interest for biologists – a yellow-necked field mouse (*A. flavicollis*) and a long-tailed field mouse (*A. sylvaticus*), because of their sympatry, high morphological similarity and difficult discrimination between each other. Their geographical areas overlap, both species share similar way of life, and they also possess similar karyotypes (Zima and Král, 1984). *A. flavicollis* is usually bigger than *A. sylvaticus*, but in southern parts of its habitat the reverse clinal variations in body size and colour of the fur appear (Filippucci et al., 1984). These two species are characterized by a complex of genetic differentiation (Michaux et al., 2003; Hoofer, 2007; Bugarski-Stanojević et al., 2011), majority of which is supposed to have occurred in the Quaternary (Hewitt, 2001). *A. sylvaticus* survived last glaciation (22-16 kya) in the Iberian peninsula, whereas *A. flavicollis* survived in Balkan, where *A. sylvaticus* underwent a severe genetic bottleneck (Michaux et al., 2005). The differences can be also influenced by interspecific competition, because both species live in sympatry.

1.3.2. Rodentia: Arvicolidae: Clethrionomys and Microtus

Within Arvicolidae, the subfamily Arvicolinae is a species-rich, monophyletic group, comprising 151 species in 28 genera (Musser and Carleton, 2005). The genus *Microtus* (meadow voles) encompasses 60 species spread throughout the whole Palearctic and Holarctic (Chaline et al., 1999). However, the classification of this genus into subgenera and species is complicated due to its

variability caused by rapid divergence (Gromov and Polyakov, 1977; Zagorodnyuk, 1990; Meyer et al., 1996; Bastos-Silveira et al., 2012). The species richness of the genus *Microtus* is caused by a recent ongoing radiation (Jaarola et al., 2004). Similarly, taxonomy and nomenclature of *Clethrionomys* have been unstable for decades. Musser and Carleton (2005) used the name *Myodes* for the red-backed vole, which has become widespread. Nevertheless, the current valid name is *Clethrionomys* (Tesakov et al., 2010).

During the Quaternary period, the distribution range of Palearctic species changed significantly (Webb and Bartlein, 1992). Central Europe was steppe-tundra, while broadleaved forests drifted to Mediterranean peninsulas (Blondel, 1995). Several populations of *Clethrionomys glareolus* (bank voles) moved to southern broadleaved habitats, survived there, and after glacial maxima returned back to recolonize central Europe (Taberlet et al., 1998; Hewitt, 1999, 2001; Michaux et al., 2003). Species from eastern or northern refugia also participated in this recolonization (Jaarola et al., 1999; Jaarola and Searle, 2002; Brunhoff et al., 2003). Currently, *Clethrionomys glareolus* is dispersed across various geographic zones, from Mediterranean to beyond the polar circle and from British Islands and northern Spain to Siberia (Cook et al., 2004; Amori et al., 2008). It occurs in variety of forest habitats, shrubs or groves (Kotlík et al., 2006). It has both diurnal and nocturnal activity and does not hibernate during the winter (Klimpel et al., 2007). All members of the tribe *Arvicolini* possess various adaptations to their food demands and the subterranean way of life. Abundance of vole populations fluctuates in specific cycles (3 years for both *C. glareolus* and *Microtus* spp.), but it can be also influenced by climatic changes (Brommer et al., 2010). This cyclic population dynamics is an important feature for animals living in boreal habitats (Lindström et al., 2001), and it can significantly influence the food chains in these areas, mainly for their predators (Linden, 1988; Ims and Fuglei, 2005).

The beginning of the differentiation of the genus *Microtus* was 2 mya (Chaline et al., 1999), and it has become one of the most diverged genus with rapid speciation, comprising 60 extant species. According to Triant and DeWoody (2006), a new species of *Microtus* emerges approximately every 30 000 years, which is unique in vertebrates. The nature of this peculiarity is still debated, but it may be caused by karyotype differentiation (2n = 17-64) (Triant and DeWoody, 2006).

Phylogeny of voles was for a long time based solely on morphology of recent and fossil individuals (Chaline et al., 1999; Ledevin, 2010). However, recent analyses have focused on molecular markers, such as DNA/DNA hybridization (Catzeflis et al., 1987), LINE-1 (Modi, 1996), mtDNA and rDNA (Suzuki et al., 1999, Martin et al., 2000; Conroy et al., 2001; Cook et al., 2004; Jaarola et al., 2004), or LCAT and vWF (Michaux et al., 2001).

The subfamily Arvicolinae is monophyletic (Musser and Carleton, 2005; Buzan et al., 2008). Tribus *Lemmini* is supposed to be the most basal group of voles and a sister group to the remaining members of the subfamily Arvicolinae, as supported also by morphology (Hinton, 1926; Gromov and Polyakov, 1992). All the tribes are monophyletic, except of *Arvicolini* which seem to be paraphyletic due to the uncertain position of *Arvicola terrestris* (Niethammer and Krapp, 1982; Catzeflis et al., 1987; Chaline and Graf, 1988).

In this study, I focused on rodent-coccidia host-parasite model system. The aim of this study is twofold: to place rodent-specific eimerians into the phylogenetic tree of *Eimeria* and to study for the first time genealogy and population structure of these rodent parasites.

2. The aims of the study

The main goal of this study was to analyse phylogenetic relationships and population structure of coccidia parasitizing rodents of the genera *Apodemus*, *Clethrionomys* and *Microtus,* sampled from different localities across Europe.

In order to achieve the aims mentioned below, an array of techniques of field and laboratory parasitology, together with methods of molecular phylogenetics and population genetics were employed during the course of my master study.

Particular aims can be defined as follow:

- To determine the prevalence of coccidia infecting above mentioned rodents in different countries/localities in Europe, and to analyse the spectrum of species of obtained *Eimeria*.
- To study evolutionary relationships (interspecific and intraspecific) between obtained coccidia and their rodent hosts.
- To reconstruct the genealogy and population structure of eimerian parasites infecting field mice of the genus *Apodemus*.
- To analyse the influence of host specificity and biogeography on the population structure and speciation of *Eimeria*.
- To determine the critical elements of biogeography and host distribution (geographic areas and host species) of *Eimeria* that need to be sampled in further studies.

3. Materials and methods

3.1. Field collections and origin of samples

Field collections were carried out in the course of 2006-2012, under official permits from the Office for the South Bohemian Region, Department of the Environment, Agriculture and Forestry (Permit Number: KUJCK 11134/2010 OZZL/2/Ou) and the Ministry of the Environment of the Czech Republic (Permit Number: 27873/ENV/11); the protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia (Permit Number: 13841-11). During this period, in total 2276 small mammals were collected from 14 states in Europe (1898 of these samples were collected during my involvement in the study in the years 2009-2012). The list of localities and collected species is provided in Tables 1, 2 and Figure 3. Faeces from each individual animal were collected and kept in 4% potassium dichromate ($K_2Cr_2O_7$) solution. Host tissues (a small piece of ear or tail) were preserved in absolute ethanol for PCR determination.

3.2. Coprological examination and oocyst morphology

The presence of parasites in collected faeces was examined microscopically by flotation in the Sheather's sucrose solution of the density 1.30 (Duszynski and Wilber, 1997; Zajac and Conboy, 2006). Determination of coccidian species/morphotypes was based on morphology and morphometry of sporulated oocysts, according to guidelines published by Duszynski and Wilber (1997).

3.3. DNA extraction, PCR amplification of selected genes, sequencing

Eimerian DNA from positive faecal samples was isolated with FastDNA ® SPIN for Soil Kit (MP Biomedicals) according to manufacturer's instructions. For amplification, a mitochondrial gene for cytochrome c oxidase subunit I (COI), which is a good marker for intraspecific and interspecific variability, and a nuclear 18S rRNA gene (SSU gene), were selected. Specific primers for amplification of ~800 bp COI were designed according to published sequences of *Eimeria* species and related coccidians in the GenBank (NCBI) (forward primer: 5′-GGTTCAGGTGTTGGTTGGAC-3′, reverse primer: 5′-ATCCAATAACCGCACCAAGAG-3′). For amplification of ~1300 bp of 18S rDNA, specific primers were adopted from Kvičerová et al. (2008) (forward primer: 5′-GAAACTGCGAATGGCTCATT-3′, reverse primer: 5′-CTTGCGCCTACTAGGCATTC-3′). HotStarTaq DNA Polymerase (Qiagen) was used for all PCR

reactions. PCR products were enzymatically purified and directly sequenced; five independent PCR products were sequenced for each sample. When needed, PCR products were cloned into the pGEM–T Easy Vector (Promega) and plasmids were then extracted by the PureLink Quick Plasmid Miniprep Kit (Invitrogen); five plasmids for each sample were then sequenced. Samples were sequenced by Macrogen, Inc. (Amsterdam, the Netherlands) on an automatic 3730XL DNA analyzer.

3.4. Sequence assembling, alignments and phylogenetic analyses

Obtained sequences were identified by BLAST (www.ncbi.nlm.nih.gov) and assembled using the Sequence Scanner v.1.0 (Applied Biosystems), EditSeq 5.05 and SeqMan 5.05 (DNASTAR Inc.) programs. Alignments were created in BioEdit program v.7.0.5.3. (Hall, 1999) by the ClustalW algorithm (Thompson et al., 1994) and adjusted manually. 18S rDNA sequences were aligned in the nucleotide mode, COI sequences were aligned in the amino acid mode, then switched to nucleotide mode and used for the analyses. Phylogenetic relationships were analysed by three approaches - maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), employing three different phylogenetic programs - PAUP v.4b10 (Swofford, 2002), PHYML v2.4.3s (Guindon and Gascuel, 2003) and MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001) (for details, see Table 3). Final trees were visualized by the TreeView v. 1.6.6. program (Page, 1996). COI sequences were collapsed into haplotypes and the population structure (haplotype networks, number and frequencies of haplotypes) was evaluated using the program TCS (Clement et al., 2000). Trees and haplotype networks were graphically adjusted in Adobe Illustrator CS5 v.15.0 (Adobe Systems Inc.).

4. Results

4.1. Field data

In the period of 2006-2012, in total 1627 individuals of *Apodemus* spp. were collected from 14 countries in Europe (Bulgaria, Croatia, Czech Republic, Germany, Great Britain, Greece, France, Italy, Macedonia, Poland, Slovakia, Slovenia, Spain and Turkey). Till now, 291 samples were *Eimeria* positive. Samples were collected at 18 localities in the Czech Republic (No. 1-18 in the Figure 3), 6 localities in Slovakia (No. 19-24) and 16 localities in other European countries (No. 25-40) (Figure 3).

During the course of the study, 466 individuals of *Clethrionomys glareolus* and 183 individuals of *Microtus* spp. were collected from 9 European countries (Croatia, Czech Republic, Germany, Great Britain, France, Italy, Macedonia, Poland and Slovakia) (Figure 3). The number of *Eimeria*-positive samples was significantly lower than number of positive samples from *Apodemus* spp. (only 42 *Eimeria*-positive samples from *C. glareolus* and 36 from *Microtus* spp. were found).

Table 1. Summary of trapped and infected *Apodemus* spp., with number of obtained sequences.

country	trapped	positive	prevalence	COI	SSU
Bulgaria	43	4	9,3%	0	0
Croatia	102	14	13,7%	0	0
Czech Republic	574	100	17,4%	40	23
France	21	3	14,3%	1	0
Germany	209	42	20,1%	7	6
Great Britain	28	6	21,4%	2	2
Greece	9	0	0%	0	0
Italy	86	5	5,8%	6	4
Macedonia	63	10	15,9%	4	2
Poland	74	23	31,1%	0	0
Slovakia	381	84	22,0%	23	14
Slovenia	4	0	0%	0	0
Spain	20	0	0%	0	0
Turkey	13	0	0%	0	0
Total	**1627**	**291**	**17,9%**	**83**	**51**

Table 2. Summary of trapped and infected Arvicolidae with number of obtained sequences.

country	trapped		positive		prevalence		COI		SSU	
	CG	MI	CG	MI	CG	MI	CG	MI	CG	MI
Croatia	11	8	0	0	0%	0%	0	0	0	0
Czech Republic	223	136	19	36	8,5%	26,5%	3	4	1	2
France	1	1	0	0	0%	0%	0	0	0	0
Germany	107	4	15	0	3,7%	0%	4	0	2	0
Great Britain	1	1	0	0	0%	0%	0	0	0	0
Italy	7	0	1	0	14,3%	0%	1	0	1	0
Macedonia	7	1	0	0	0%	0%	0	0	0	0
Poland	39	4	5	0	12,8%	0%	0	0	0	0
Slovakia	70	28	2	0	2,3%	0%	0	0	0	0
Total	**466**	**183**	**42**	**36**	**9,0%**	**19,7%**	**8**	**4**	**4**	**2**

CG, *Clethrionomys glareolus*; MI, *Microtus* spp.

4.2. Molecular data

I obtained 83 *Eimeria* sequences of the COI gene and 51 *Eimeria* sequences of the SSU gene from *Apodemus* spp. (see Table 1), and 12 *Eimeria* sequences of COI gene together with 6 sequences of SSU gene from the family Arvicolidae (see Table 2). Furthermore, I obtained one COI sequence of *Isospora* sp. from *A. flavicollis*, and several *Eimeria* sequences from other small mammals (*Crocidura* sp., *Marmota marmota*, *Mus musculus*, *Neomys fodiens* and *Sorex* sp.). These sequences were included into the analyses to improve the sample background. The length of sequences varied between 303-804 bp for the COI gene (mean ~700 bp), and 522-1417 bp for the SSU gene (mean ~1200 bp). GC content was ~35% for the COI gene, and ~47% for the SSU gene.

Table 3. Information on phylogenetic analyses of molecular data and parameters used.

Matrix	MP (PAUP)	ML (Phyml)	BI (MrBayes)
18S rDNA 99 sequences, alignment length 1377 bp	hsearch + TBR 1000 replicates best tree = 681, CI = 0,5991	GTR + Γ +I 1000 replicates -ln: 5924.818956	GTR + Γ +I mcmc = 10,000,000 gens. burn-in = 1100 trees
COI 114 sequences, alignment length 720 bp	hsearch + TBR 1000 replicates best tree = 722, CI = 0,4654	GTR + Γ +I 1000 replicates -ln: 4498.179460	GTR + Γ +I mcmc = 10,000,000 gens. burn-in = 2000 trees

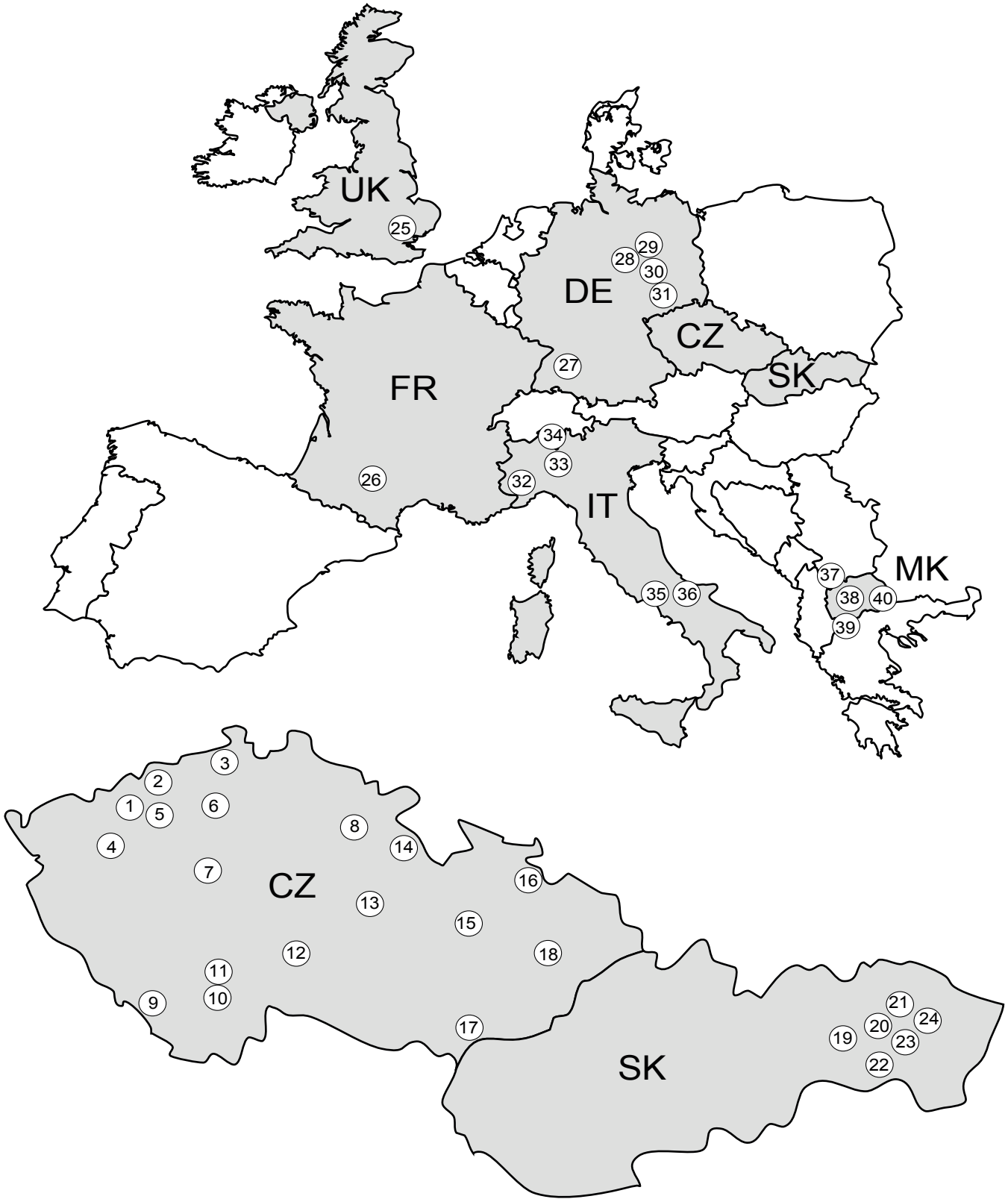

Figure 3. Map of the sampled localities.
Czech Republic (CZ): 1-Vykmanov and Klášterec nad Ohří, 2-Litvínov, 3-Pastýřské Kameny, 4-Stružná, 5-Lestkov, 6-Solany, 7-Křivoklát, 8-Chotěborky, 9-Borová Lada, 10-Boršov nad Vltavou, 11-Vomáčka u Zlivi, 12-Zajíčkov, 13-Jimramov, 14-Sedloňov, 15-Velký Kosíř, 16-Cvilín, 17-Nesyt, 18-Rajnochovice; **Slovakia (SK)**: 19-Hýľov, Hlboká dolina, 20-Botanická záhrada Košice, 21-Anička–Košice, 22-Šebastovce, 23-Nižné Kapustníky, 24-Rozhanovce; **England (UK)**: 25-Ashford; **France (FR)**: 26-Toulouse; **Germany (DE)**: 27-Baiersbronn, 28-Sollichau, 29-Torgau, 30-Lausa, 31-Pinkowitz; **Italy (IT)**: 32-Valdieri, 33-Bubbiano, 34-Brinzio, 35-Forli del Sannio, 36-Civitanova del Sannio; **Macedonia (MK)**: 37-Popova Šapka, 38-Krusevo, 39-Nižepole, 40-Belovodica.

4.3. Phylogenetic relationships

Phylogenetic analyses of both genes split the sequences of *Eimeria* infecting field mice into 5 lineages (I-V; Figures 4-7). Lineage I is a robust and monophyletic branch, phylogenetically isolated from the rest of the lineages. It consists of samples from various localities across the Bulgaria, Czech Republic, Germany, Great Britain, Italy, Macedonia and Slovakia. According to morphology and morphometry of sporulated oocysts, its morphotype corresponds to *Eimeria jerfinica* (Figure 8). The four remaining lineages form a single large cluster (Figures 4-7). The lineage II is similar to the lineage I in a broad host range and geographic distribution. It occurs across 5 of the studied countries and all studied hosts of the genus *Apodemus*. Morphotype of this coccidium is *Eimeria apionodes*. Eimerians from the lineage III were found rarely, only in few samples, and it is therefore difficult to describe its distribution pattern with certainty. In the continental Europe, the lineage is specific for *A. flavicollis*, while in Great Britain it switched to *A. sylvaticus* (Figure 8). It corresponds to the morphotype of *E. kaunensis*, possessing an oocyst residuum, in contrast to all other lineages. Lineage IV is formed by *Eimeria* parasitizing exclusively on *Apodemus agrarius*. This lineage was found only in eastern Slovakia. It possesses morphological traits corresponding to *E. alorani*. Lineage V is dispersed across 5 states. Its morphotype is *E. apionodes*. It was found in *A. flavicollis*, *A. sylvaticus* and *Apodemus* sp., so it has very similar pattern as lineage II. However, it was not found in *A. agrarius* (Figure 8). Three sequences (AF 5, AGR 21831 and AS 77) are completely different and do not cluster into any of the above mentioned lineages. Similarly, *Eimeria* sequences from Arvicolidae are spread across the whole tree, sometimes placed as isolated single-branches outside the clusters (Figures 4-7, 9).

Eimerian lineages obtained by phylogenetic analyses are compatible with the haplotype networks constructed in TCS program. When collapsed into haplotypes, the 114 COI sequences generated 2 distinct haplotype networks with 25 unique haplotypes (Figure 10). The smaller network is formed by sequences corresponding to the lineage I (Figures 8 and 10). This network is well-structured and contains only few missing nodes. The second network corresponds to the lineages II-V and is highly diversified. Most of the branches correspond to individual lineages obtained by the phylogenetic analyses. The lineage II of the haplotype network splits into 2 parts – one is composed of 4 sequences from *A. agrarius*, and the second bigger part includes eimerians from *A. sylvaticus*, *A. flavicollis* and *Apodemus* sp. Half of the sequences from Arvicolidae form a single cluster, while the rest are set aside separately as individual independent haplotypes. Several sequences (AF B13, AGR 21831, ITSA1, AF 5, AS 77, MA 98) formed distinct separate clades in the TCS analysis (Figure 10).

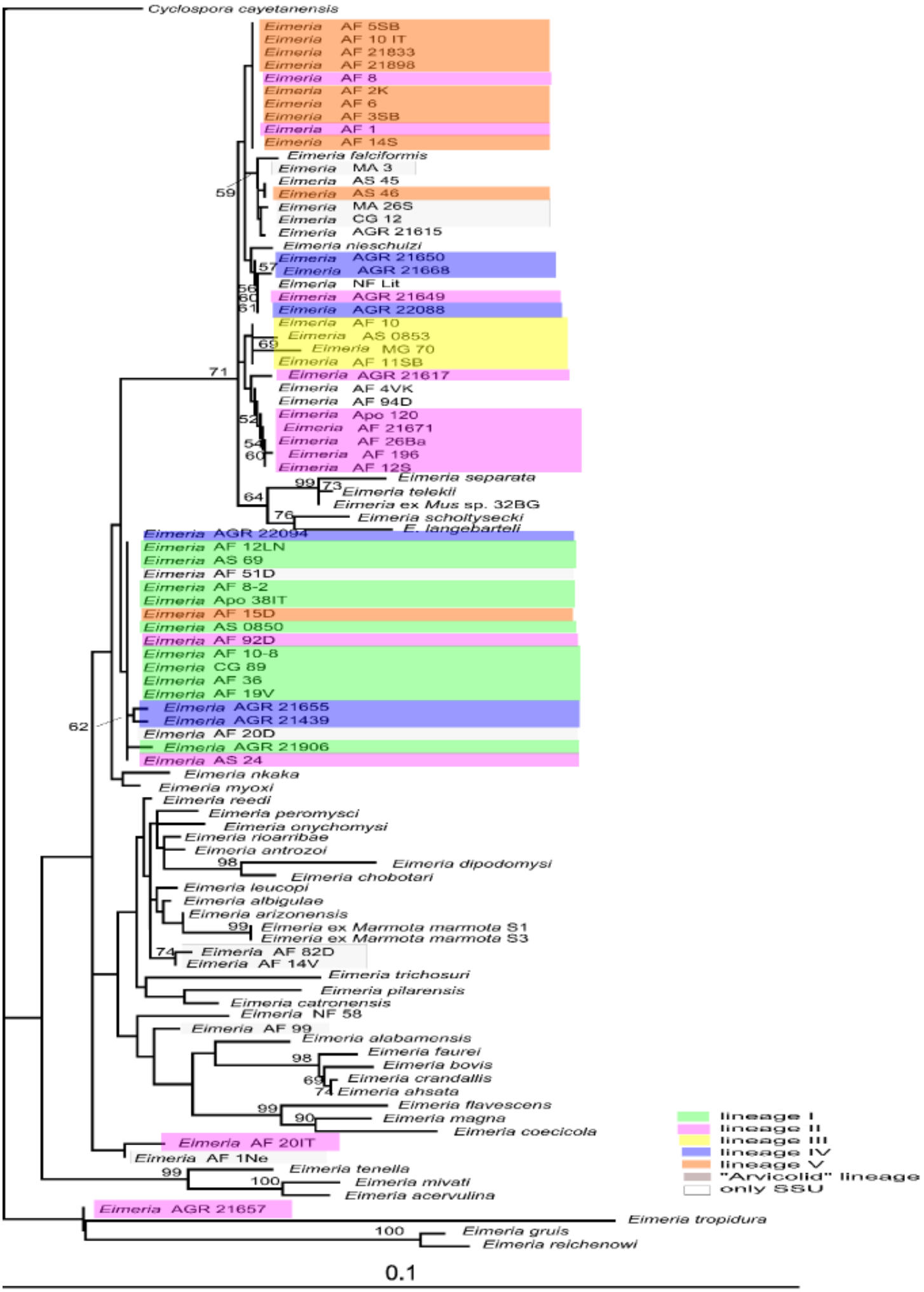

Figure 4. Maximum likelihood tree inferred from sequences of SSU.
The tree is rooted with *Cyclospora cayetanensis*. Numbers at the nodes show bootstrap values higher than 50 %. Geographic origin and host species are listed in Supplement. The same branching pattern was obtained also by MP analysis.

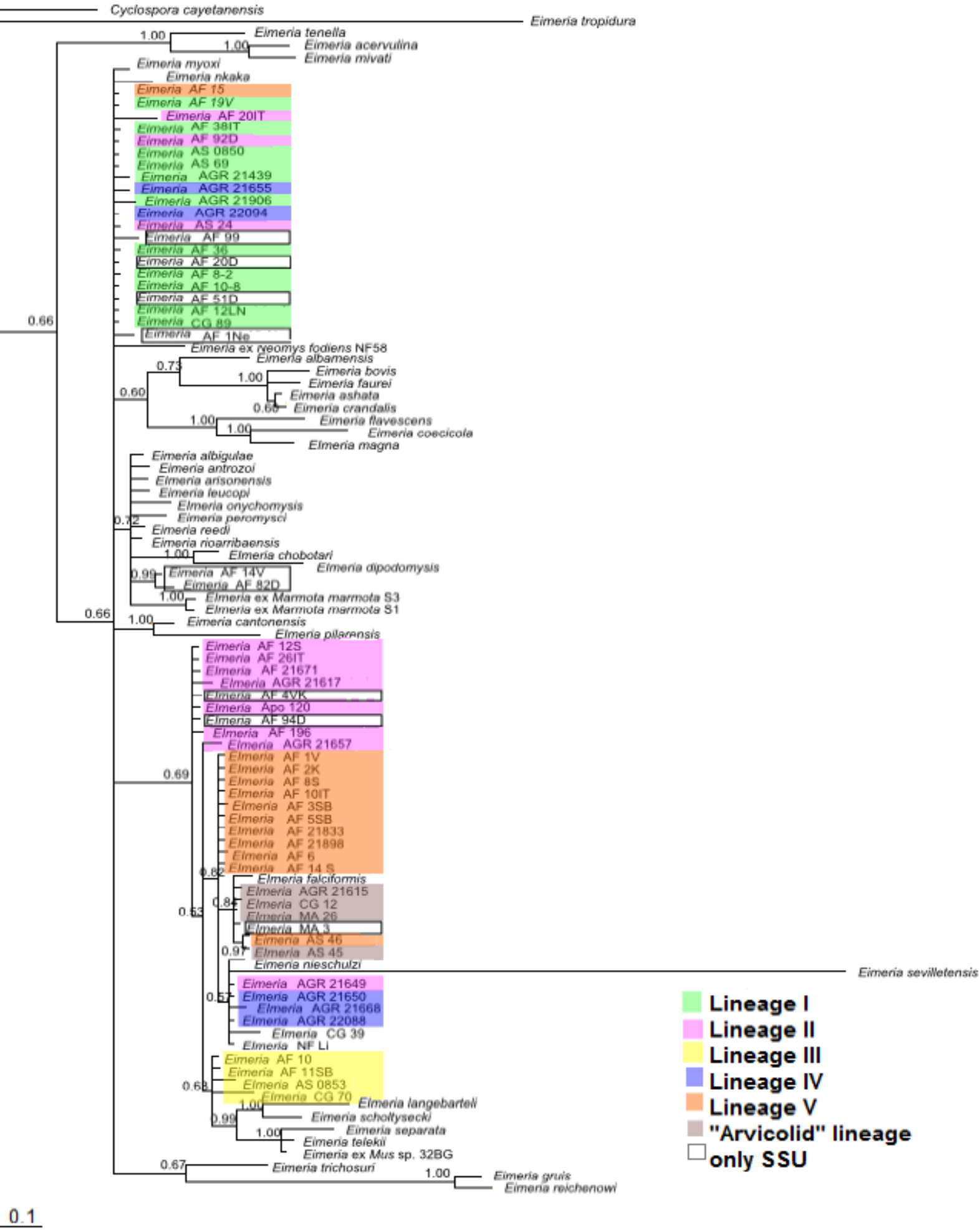

Figure 5. Bayesian inference tree inferred from sequences of SSU.
The tree is rooted with *Cyclospora cayetanensis*. Numbers at the nodes show posterior probabilities higher than 0.50. Geographic origin and host species are listed in Supplement.

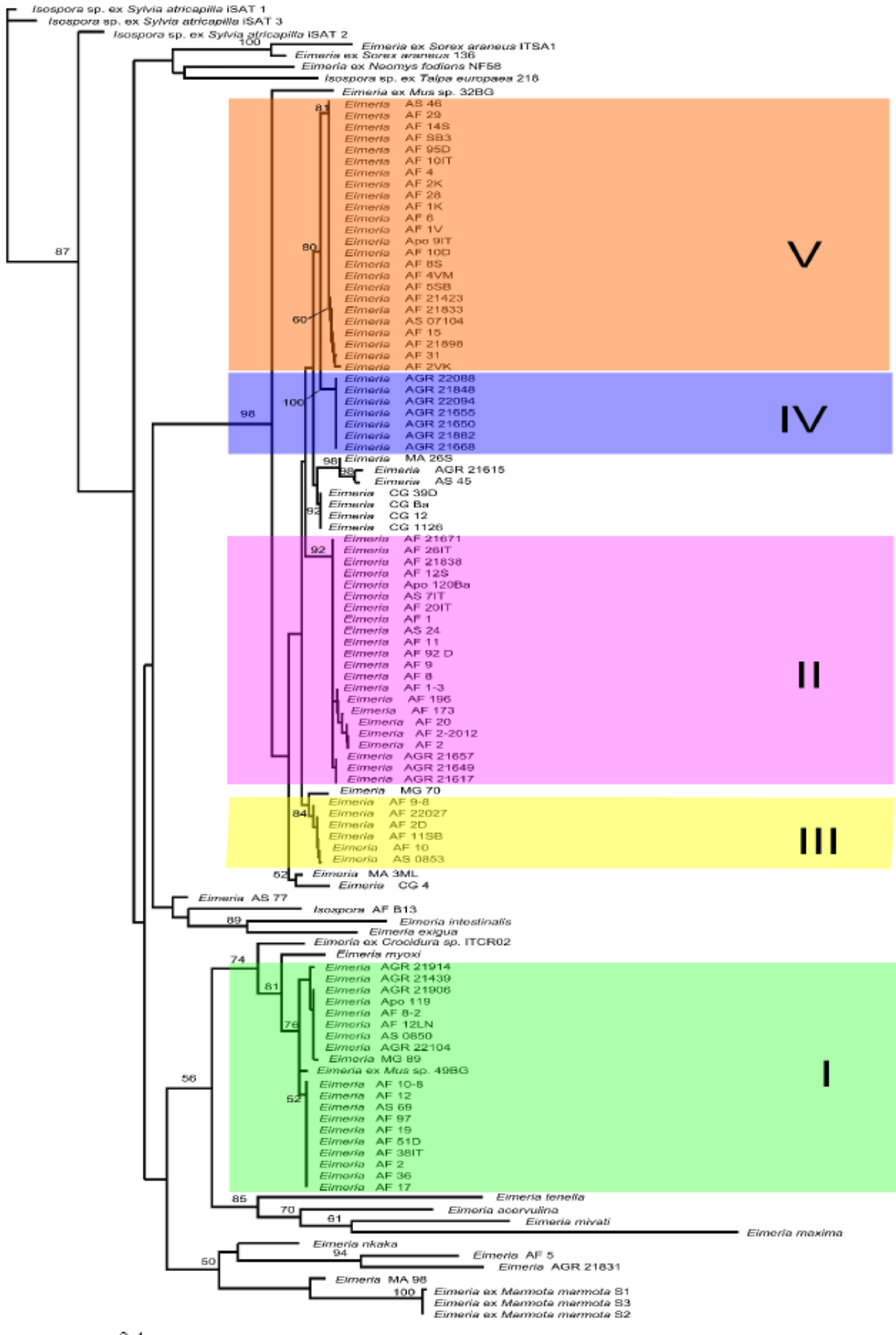

Figure 6. Maximum likelihood tree inferred from sequences of COI.
The tree is rooted with *Isospora* sp. ex *Sylvia atricapilla* iSAT1. Numbers at the nodes show bootstrap values higher than 50 %. Geographic origin and host species are listed in Supplement. The same branching pattern was obtained also by MP analysis.

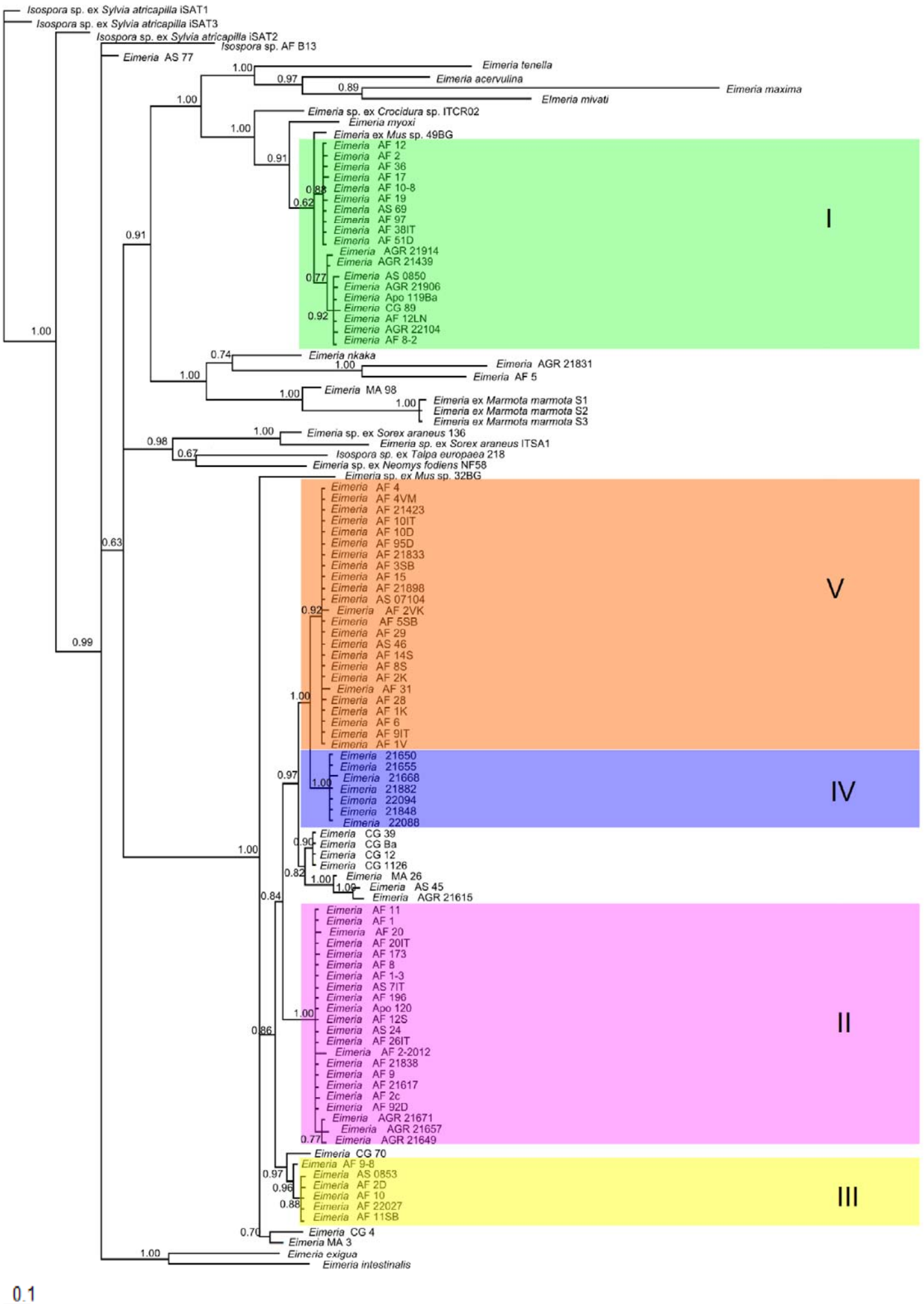

Figure 7. Bayesian inference tree inferred from sequences of COI.
The tree is rooted with *Isospora* sp. iSAT1. Numbers at the nodes show posterior probabilities higher than 0.50. Geographic origin and host species are listed in Supplement.

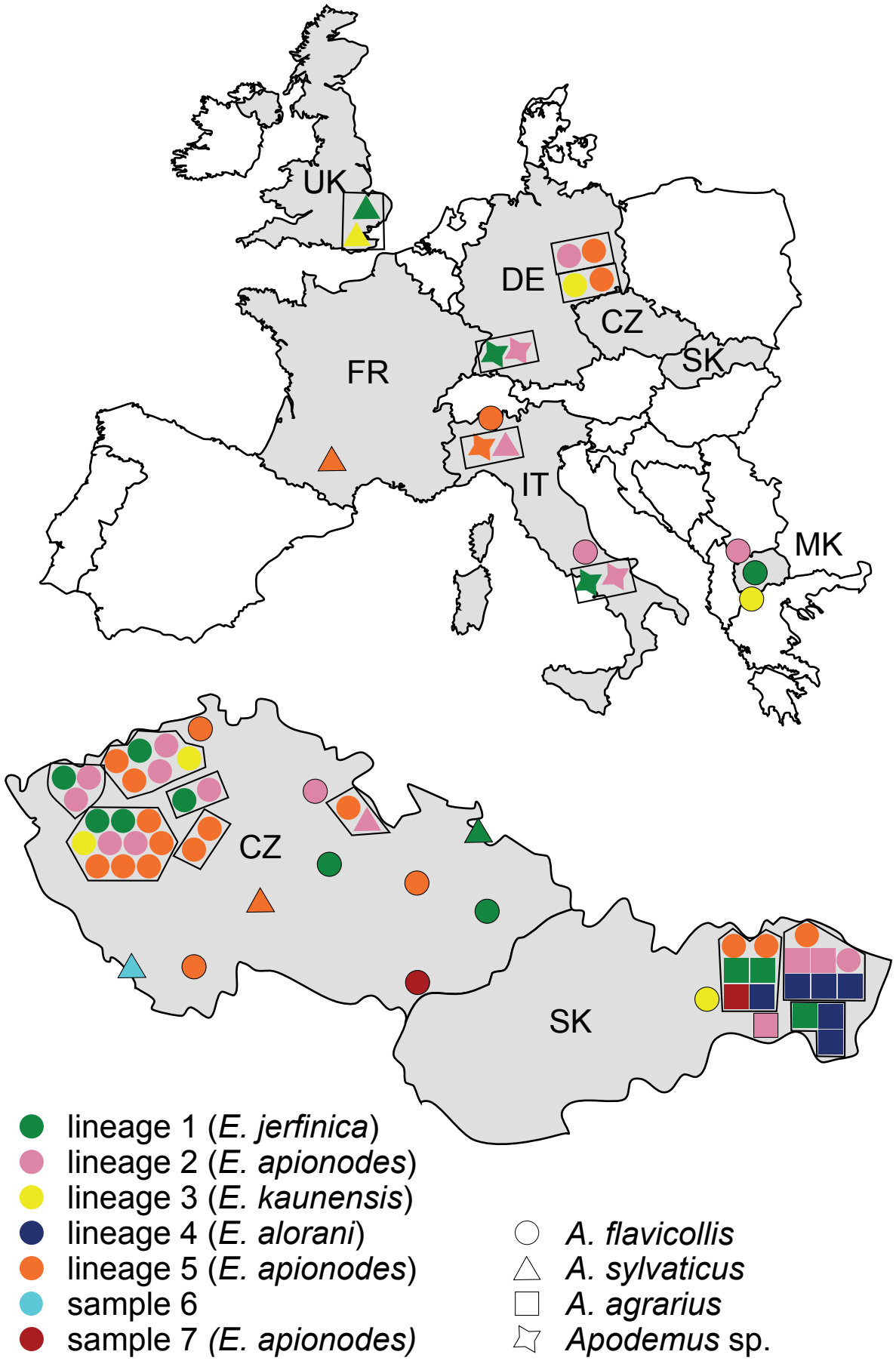

Figure 8. Map of the obtained phylogenetic lineages (*Eimeria* spp. from *Apodemus* spp.).

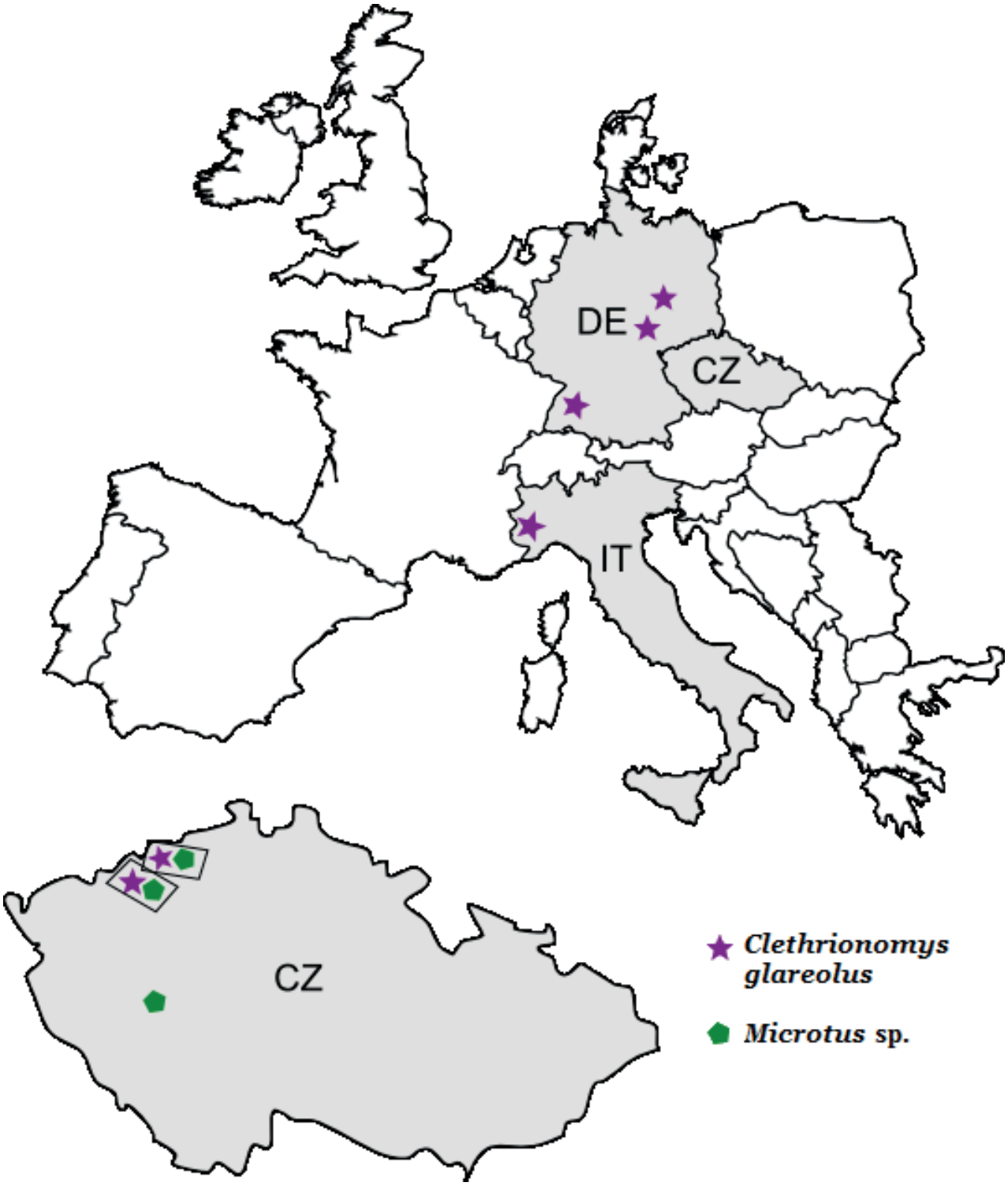

Figure 9. Map of the obtained phylogenetic lineages (*Eimeria* spp. from arvicolid rodents).

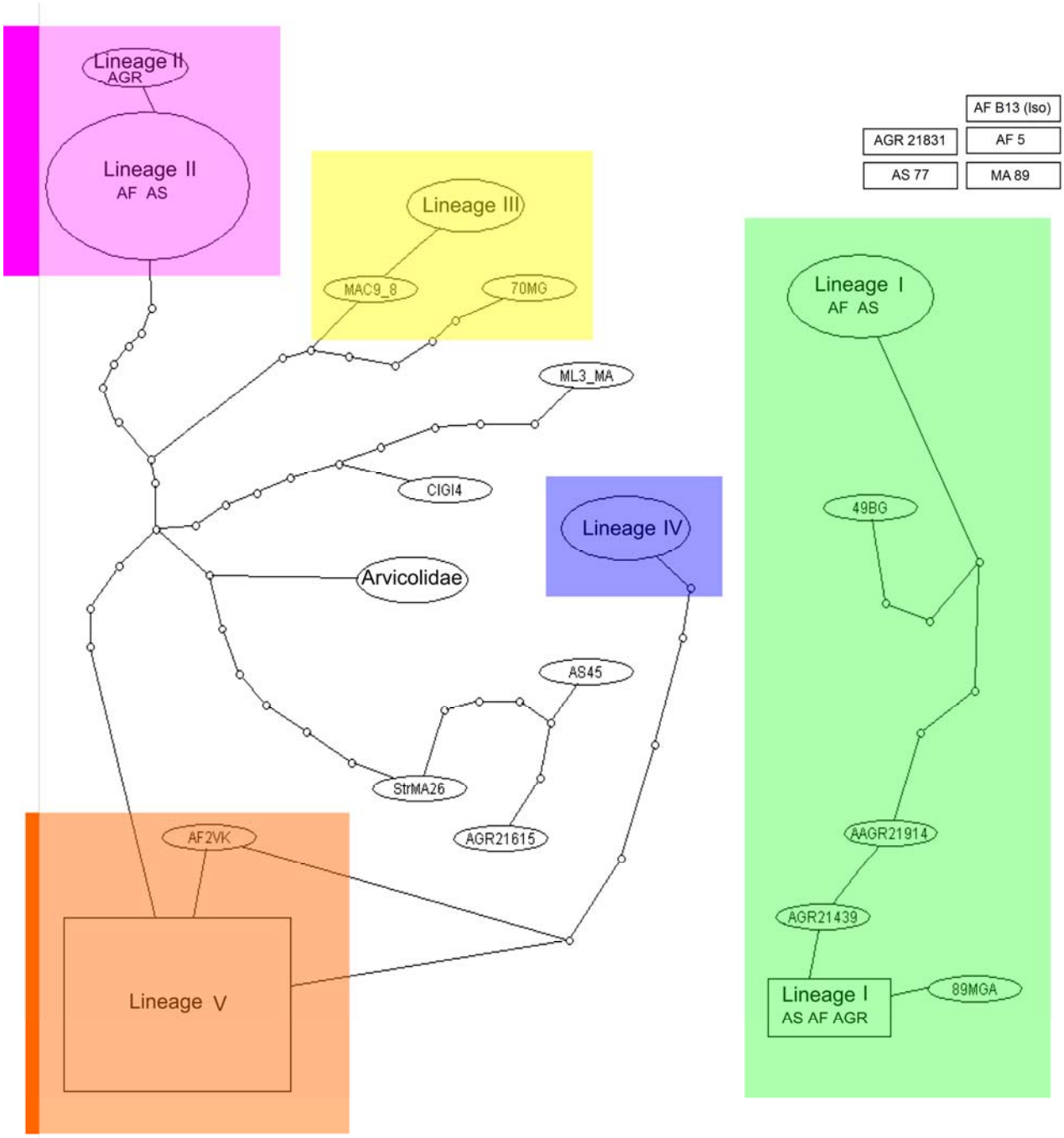

Figure 10. Haplotype networks of 114 *Eimeria* specimens obtained by TCS analysis. Geographic origin and host species are listed in Supplement.

5. Discussion

5.1. Biological diversity and coevolutionary patterns

The most remarkable feature obvious from our analyses is the biological variability among the obtained lineages. While some of them encompass samples from wide geographic distribution and host spectra (lineages I and II), others contain only parasites from a single host species or restricted geographic area (lineage IV). Similar flexibility has been shown in few other parasites. For example, the strong differences in host specificity and geographic distribution were reported in the lice *Polyplax serrata* infecting *Apodemus* species (Štefka and Hypša, 2008), or in the tapeworm *Ligula intestinalis* infecting water birds (Štefka et al., 2009).

The obtained sequences form a complex pattern of phylogenetic and genealogical relationships within *Eimeria* species/morphotypes. Most of the sequences are placed into *Apodemus*-specific clusters. Individual lineages recognized within these clusters are mostly genus-specific rather than species-specific. This host-determined clustering is however not universal, three sequences (AF 5, AGR 21831 and AS 77) were placed outside the clusters, and show high differences in comparison to the major *Apodemus*-specific branches.

Individual lineages well demonstrate several strikingly different patterns of the host specificity and geographic distribution. The lineages I, II and V do not show any clear host specificity, neither restricted geographic distribution within the sampled area. They parasitize on various host species and are widely dispersed almost across all studied countries. However, despite this similarity, their host ranges show a difference which at this stage of sampling and analysis can be also expressed with some caution and degree of uncertainty: the lineages II and V consist exclusively of sequences from *Apodemus* species (which is also in agreement with the coccidian morphotype *E. apionodes*, typical for this genus), while the phylogenetically distant lineage I also contains eimerians from other rodent genera. Apart from these lineages, the *E. apionodes* morphotype has been observed also in the samples AF 5 and AGR 21831, from two different localities, which have been placed outside the main five lineages.

Lineages II-V form a large cluster that seems to be monophyletic, but not *Apodemus*-specific. It consists of samples from all studied members of the genus *Apodemus* and family Arvicolidae. This poses a question whether eimerian samples from Arvicolidae are regular infections or may only represent a passage through the digestive system of Arvicolid rodents. Experiments performed by several authors (de Vos, 1970; Upton et al., 1992; Šlapeta et al., 2001; Čížkovská, 2003; Kvičerová et al., 2007) showed that in the non-susceptible host animal, the coccidium does not invade

intestine, but is digested by the host and is not discharged in faeces. This finding indicates that the former hypothesis, i.e. the regular infection, is more likely an answer to this issue.

The dispersion of the lineages indicates that all of them came from alpine glacial refugia, although the lineage IV differs from the others, being distributed only in the eastern part of Slovakia (where is the most western border of its occurrence). However, phylogenetic analyses placed it into the cluster of lineages II–V, which indicates a common history in the same refugium. Peculiarity of the lineage IV is given also by the species composition – it is formed exclusively by eimerians infecting *A. agrarius.* Moreover, this lineage of *Eimeria* possesses morphological traits corresponding to *E. alorani*, which has not been well-studied yet. It was described for the first time by Hůrková et al. (2005) from *Apodemus mystacinus* from Jordan.

In addition to the obvious non-monophyly of the *Apodemus*-derived eimerians (i.e. lineage I vs. the cluster II-V), the complete sample from *Apodemus* shows even stronger phylogenetic variability: three sequences do not cluster into any of the above mentioned lineages. Similarly, eimerian sequences from Arvicolidae are spread within the whole tree. Part of these sequences form a small lineage inside the cluster formed by lineages II –V. I also obtained a single sequence of *Isospora* sp. (sample No. AF B13) from Litvínov (Czech Republic). Species of the genus *Isospora* are divided into 2 lineages according to the host specificity; *Isospora* spp. from birds form their own cluster, whereas *Isospora* spp. infecting mammals cluster within *Eimeria* species (Franzen et al., 2000). I proved this fact also in our analyses.

Apart from these broadly distributed lineages (in respect to the host species and geography), the cluster II-V also contains lineages with peculiar traits and patterns of distribution. Lineage III is represented by the morphotype *E. kaunensis*, possessing an oocyst residuum. This position is quite unexpected in respect to the hypothesis of existence of two distinct rodent *Eimeria* lineages of (Reduker et al., 1987; Hnida and Duszynski, 1999a, b; Zhao and Duszynski, 2001b). However, in our analyses, the only lineage possessing the OR branches within the whole cluster lacking this structure. Another peculiarity of this lineage is the low number of samples found across the whole sampled area: it is created only by few sequences, but covers 5 different countries. It is also remarkable that in the continent this lineage is specific to *A. flavicollis*, but in Great Britain it seems to have switched to *A. sylvaticus*. However, it is difficult to determine whether this finding shows on a sparse population density and a host switch, until more samples are obtained and analysed.

The results of genealogical analysis obtained by the program TCS correspond to results of phylogeny. The only interesting finding is that the last branch composed of lineages IV and V is connected by sample AF 2 VK. In phylogenetic analyses, this sample clustered into lineage V, whereas here is excluded outside the group formed by all sequences from that lineage.

5.2. Future prospects

Part of the obtained samples contained mixed infections, composed of more than one *Eimeria* morphotype (usually two to three). Using the molecular techniques mentioned in Materials and Methods, I always managed to isolate and sequence only one genotype. Searching for new methods allowing to obtain all genotypes from a mixed infection is one of our future goals.

Other target is additional sampling, mainly in unexplored localities or along the borders of studied localities to get better knowledge on possible origin of individual lineages (e.g. field collections in Ukraine, Hungary, Poland and Russia to obtain more information on distribution of the lineage IV), or to resolve the phylogenetic status of samples AF5 and 77AS, which are single samples from 2 different localities.

Although the sampling of rodents of the family Arvicolidae was of a reasonable size, we obtained only few sequences, because the prevalence of *Eimeria* in members of this family was significantly lower than in *Apodemus* spp. There is a question whether the absence of coccidia in *Microtus* hosts in Europe reflects real biological circumstances, or if it is only an artifact due to the low sampling. It would be interesting to find the answer by more intensive sampling.

In the phylogenies presented here, eimerian parasites from several hosts evolutionary related to the genus *Apodemus* that could potentially break the monophyly of some of our lineages, are missing (e. g. *Micromys* or *Rattus*) (Martin et al., 2000; Michaux et al., 2002). With current amount of data, it is not possible to conclude whether the lineages represent genetically separated species or just local populations. Nevertheless, it is apparent from the phylogenetic arrangement and sequence similarities that at least the lineages II to V are closely related and could serve as a suitable model for investigating adaptive processes in parasites during their speciation.

6. Conclusion

In this master thesis, I studied phylogenetic relationships and population structure in host-parasite model of *Eimeria* and small rodents (*Apodemus* and Arvicolidae) based on the nuclear and mitochondrial genes (95 COI and 56 SSU sequences). I revealed 5 genetic lineages displayng diverse patterns of relationships between the phylogeny and biology. They mostly possess broader host- and geography- distribution, except of a single lineage (No. IV), which was restricted only to *A. agrarius* in eastern Slovakia. All of the lineages lack the oocyst residuum, except for the lineage III, which is not phylogenetically distant but branches within the wide cluster of the other lineages. Because of intensive sampling, our analyses are robust and reliable. However, additional collections and studies are needed to precise further some of the obtained patterns, characters, and phenomena.

Results of this Master study are currently being prepared for publication "Population structure, host specificity and biogeography in *Apodemus* and *Eimeria* host-parasite model system" to be submitted in a parasitological journal.

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8. Supplement

List of samples, their origin, and obtained sequences.

(BG – Bulgaria, CZ – Czech Republic, DE – Germany, FR – France, IT – Italy, MK – Macedonia, SK – Slovak Republic, UK – England; x – obtained, - failed, L – arvicolid lineage)

Apodemus agrarius (16 COI and 11 SSU sequences of *Eimeria* spp.)

Sample name	Locality	District	Country of origin	Lineage	COI	SSU
AGR 21615	Šebastovce	Košice	SK		**x**	**x**
AGR 21617	Šebastovce	Košice	SK	**2**	**x**	**x**
AGR 21439	Rozhanovce	Košice-Okolie	SK	**1**	**x**	**x**
AGR 21649	Rozhanovce	Košice-Okolie	SK	**2**	**x**	**x**
AGR 21650	Rozhanovce	Košice-Okolie	SK	**4**	**x**	**x**
AGR 21655	Rozhanovce	Košice-Okolie	SK	**4**	**x**	**x**
AGR 21657	Rozhanovce	Košice-Okolie	SK	**2**	**x**	**x**
AGR 21668	Rozhanovce	Košice-Okolie	SK	**4**	**x**	**x**
AGR 21831	Botanic Garden	Košice	SK	**-**	**x**	**-**
AGR 21848	Botanic Garden	Košice	SK	**4**	**x**	**-**
AGR 21882	Botanic Garden	Košice	SK	**4**	**x**	**-**
AGR 21906	Botanic Garden	Košice	SK	**1**	**x**	**x**
AGR 21914	Botanic Garden	Košice	SK	**1**	**x**	**-**
AGR 22104	Nižné Kapustníky	Košice	SK	**1**	**x**	**-**
AGR 22088	Nižné Kapustníky	Košice	SK	**4**	**x**	**x**
AGR 22094	Nižné Kapustníky	Košice	SK	**4**	**x**	**x**

Apodemus sylvaticus (9 COI and 6 SSU sequences of *Eimeria* spp.)

Sample name	Locality	District	Country of origin	Lineage	COI	SSU
AS 08/50	Ashford	South East	UK	**1**	**x**	**x**
AS 0853	Ashford	South East	UK	**3**	**x**	**x**
AS 07104	Toulouse	Haute-Garonne	FR	**5**	**x**	**-**
AS 7IT	Bubbiano	Milano	IT	**2**	**x**	**-**
AS 24	Sedloňov	Rychnov n. Kněžnou	CZ	**2**	**x**	**x**
AS 45	Zajíčkov	Pelhřimov	CZ	**-**	**x**	**x**
AS 46	Zajíčkov	Pelhřimov	CZ	**5**	**x**	**x**

AS 69	Cvilín	Bruntál	CZ	**1**	**x**	**x**
AS 77	Borová Lada	Prachatice	CZ	**6**	**x**	**-**

Apodemus flavicollis (53 COI and 32 SSU sequences of *Eimeria* spp., 1 COI sequence of *Isospora* sp.)

Sample name	**Locality**	**District**	**Country of origin**	**Lineage**	**COI**	**SSU**
AF 1	Solany	Litoměřice	CZ	**2**	**x**	**-**
AF 2	Solany	Litoměřice	CZ	**1**	**x**	**-**
AF 3SB	Litvínov	Most	CZ	**5**	**x**	**x**
AF 5SB	Litvínov	Most	CZ	**5**	**x**	**x**
AF 11SB	Litvínov	Most	CZ	**3**	**x**	**x**
AF 196	Litvínov	Most	CZ	**2**	**x**	**x**
AF 173	Litvínov	Most	CZ	**2**	**x**	**-**
LN 12	Litvínov	Most	CZ	**1**	**x**	**x**
AF B13	Litvínov	Most	CZ		**x**	**-**
AF 1V	Vykmanov	Chomutov	CZ	**5**	**x**	**x**
AF 2	Vykmanov	Chomutov	CZ	**2**	**x**	**-**
AF 9	Vykmanov	Chomutov	CZ	**2**	**x**	**-**
AF 19	Vykmanov	Chomutov	CZ	**1**	**x**	**x**
AF 4VM	Pastýřské kameny	Děčín	CZ	**5**	**x**	**-**
AF 8S	Stružná	Karlovy Vary	CZ	**5**	**x**	**x**
AF 8	Lestkov	Chomutov	CZ	**2**	**x**	**-**
AF 10	Lestkov	Chomutov	CZ	**3**	**x**	**x**
AF 12	Lestkov	Chomutov	CZ	**1**	**x**	**-**
AF 12S	Stružná	Karlovy Vary	CZ	**2**	**x**	**x**
AF 14S	Stružná	Karlovy Vary	CZ	**5**	**x**	**x**
AF 15	Lestkov	Chomutov	CZ	**5**	**x**	**x**
AF 17	Lestkov	Chomutov	CZ	**1**	**x**	**-**
AF 28	Stružná	Karlovy Vary	CZ	**5**	**x**	**-**
AF 29	Stružná	Karlovy Vary	CZ	**5**	**x**	**-**
AF 1K	Křivoklát	Rakovník	CZ	**5**	**x**	**-**
AF 2K	Křivoklát	Rakovník	CZ	**5**	**x**	**x**
AF 4	Boršov n. Vlt.	České Budějovice	CZ	**5**	**x**	**-**
AF 6	Boršov n. Vlt.	České Budějovice	CZ	**5**	**x**	**x**
AF 2	Vomáčka u Zlivi	České Budějovice	CZ	**1**	**x**	**-**
AF14	Vomáčka u Zlivi	České Budějovice	CZ	**-**	**-**	**x**
AF 11	Chotěborky	Trutnov	CZ	**2**	**x**	**-**
AF 31	Sedloňov	Rychnov n. Kněžnou	CZ	**5**	**x**	**-**
AF 36	Jimramov	Žďár nad Sázavou	CZ	**1**	**x**	**x**
AF 5	Nesyt	Hodonín	CZ	**7**	**x**	**-**
AF 2 VK	Velký Kosíř	Prostějov	CZ	**5**	**x**	**-**
AF 4 VK	Velký Kosíř	Prostějov	CZ	**-**	**-**	**x**
AF 97	Rajnochovice	Kroměříž	CZ	**1**	**x**	**-**

AF 99	Rajnochovice	Kroměříž	CZ	-	-	**x**
NeAF 1	Jáchymov, Dolní Žďár	Karlovy Vary	CZ	-	-	**x**
AF 21833	Botanic Garden	Košice	SK	**5**	**x**	**x**
AF 21838	Botanic Garden	Košice	SK	**2**	**x**	-
AF 21898	Botanic Garden	Košice	SK	**5**	**x**	**x**
AF 21423	Rozhanovce	Košice-Okolie	SK	**5**	**x**	-
AF 21671	Rozhanovce	Košice-Okolie	SK	**2**	**x**	**x**
AF 22027	Hýľov, Hlboká dolina	Košice-Okolie	SK	**3**	**x**	-
AF 10IT	Brinzio	Varese	IT	**5**	**x**	**x**
AF 20IT	Civitanova del Sannio	Isernia	IT	**2**	**x**	**x**
AF 26IT	Forli del Sannio	Isernia, Molise	IT	**2**	**x**	**x**
AF 38IT	Forli del Sannio	Isernia, Molise	IT	**1**	**x**	**x**
AF 2D	Pinkowitz	Meissen	DE	**3**	**x**	-
AF 10D	Pinkowitz	Meissen	DE	**5**	**x**	-
AF 20	Pinkowitz	Meissen	DE	-	-	**x**
AF 51 D	Lausa	Belgern	DE	**1**	**x**	**x**
AF 82 A/D	Sollichau	Bad Schmiedeberg	DE	-	-	**x**
AF 92 D	Torgau	Torgau-Oschatz	DE	**2**	**x**	**x**
AF 94 A/D	Torgau	Torgau-Oschatz	DE	-	-	**x**
AF 95D	Torgau	Torgau-Oschatz	DE	**5**	**x**	-
AF 1-3	Popova Šapka	Tetovo	MK	**2**	**x**	-
AF 8-2	Belovodica	Prilep	MK	**1**	**x**	**x**
AF 9-8	Nižepole (Pelister)	Bitola	MK	**3**	**x**	-
MAC 10/8	Kruševo	Krusevo	MK	**1**	**x**	**x**

***Apodemus* sp.** (4 COI and 2 SSU sequences of *Eimeria* spp.)

Sample name	**Locality**	**District**	**Country of origin**	**Lineage**	**COI**	**SSU**
Apo 119	Baiersbronn	Freudenstadt	DE	**1**	**x**	-
Apo 120Ba	Baiersbronn	Freudenstadt	DE	**2**	**x**	**x**
Apo 9IT	Bubbiano	Milano	IT	**5**	**x**	-
ItFA 38	Forli del Sannio	Isernia, Molise	IT	**1**	**x**	**x**

Clethrionomys glareolus (7 COI and 4 SSU sequences of *Eimeria* spp.)

Sample name	**Locality**	**District**	**Country of origin**	**Lineage**	**COI**	**SSU**
CG 4	Vykmanov	Chomutov	CZ	-	**x**	-
CG 12	Vykmanov	Chomutov	CZ	**L**	**x**	**x**
CG 1126	Baiersbronn	Freudenstadt	DE	**L**	**x**	-
CG Ba	Baiersbronn	Freudenstadt	DE	**L**	**x**	-
CG 39D	Lausa	Belgern	DE	**L**	**x**	**x**
CG 70	Sollichau	Bad Schmiedeberg	DE	-	**x**	**x**
CG 89	S. Anne de Valdieri	Valdieri	IT	**1**	**x**	**x**

***Microtus* sp.** (3 COI and 2 SSU sequences of *Eimeria* spp.)

Sample name	**Locality**	**District**	**Country of origin**	**Lineage**	**COI**	**SSU**
MA 98	Litvínov	Most,	CZ	-	**x**	-
MA 3ML	Litvínov	Most	CZ	-	**x**	-
MICR 3	Klášterec n. Ohří	Chomutov	CZ		-	**x**
MA 26S	Stružná	Karlovy Vary	CZ	**L**	**x**	**x**

other samples (8 COI and 5 SSU sequences of *Eimeria* spp.)

Sample name	**Locality**	**District**	**Country of origin**	**Species**	**COI**	**SSU**
NF58	Boršov n. Vlt.	České Budějovice	CZ	*Neomys* sp.	**x**	**x**
Neomys	Litvínov	Most	CZ	*Neomys* sp.	-	**x**
32BG	around Plovdiv	Plovdiv	BG	*Mus musculus*	**x**	**x**
49BG	around Plovdiv	Plovdiv	BG	*Mus musculus*	**x**	-
ITCR02	Civitanova del Sannio	Isernia	IT	*Crocidura* sp.	**x**	-
ITSA1	Brinzio	Varese	IT	*Sorex* sp.	**x**	-
S1	Gias del Prato	Valdieri	IT	*Marmota marmota*	**x**	**x**
S2	Gias del Prato	Valdieri	IT	*Marmota marmota*	**x**	-
S3	Gias del Prato	Valdieri	IT	*Marmota marmota*	**x**	**x**