Phylogeographic Analysis of Palaearctic *Ips cembrae* (Coleoptera, Scolytinae) Populations

Master thesis to obtain the academic grade

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As the poet said, 'Only God can make a tree'-
probably because it's so hard to figure out how to get the bark on.

WOODY ALLEN

ABSTRACT

Ips cembrae (Coleoptera, Scolytinae) is an economically important pest on *Larix* spp. throughout the Palaearctic. This master thesis was designed to reveal new insight into the phylogeography, phylogenetics and taxonomy of *I. cembrae*. Therefore, nine European and two Asian larch bark beetle populations were investigated for both the mitochondrial (*COI*) and the nuclear (ITS2) DNA marker. In addition, specimens were screened for possible infections with the bacterial endosymbiont *Wolbachia*, known to influence the mitogenomes of its host. A conventional PCR using *wsp* primer was performed to test for the presence of *Wolbachia*, but did not detect the endosymbiont in *I. cembrae*. Further, the beetles DNA were examined for nuclear copies of mitochondrial genes (NUMTs), which may cause artefacts in the derived genealogy. Therefore, a special DNA extraction method was applied. On the basis of the sequence chromatograms derived from this procedure, the presence of these nuclear pseudogenes could be excluded. Using the *COI* data, phylogenetic reconstruction clustered the haplotypes in two distinct clades, one clade grouping the European populations and the second clade grouping the Asian ones. European and Asian populations did not share any haplotype. Moreover, analyses revealed a high mitochondrial sequence divergence between these two geographically separated entities. Whereas a low intraspecific variation was found in European populations, results indicated a much higher genetic subdivision in Asian populations. Analyses of the ITS2 markers showed two alleles exclusively present in European populations and two alleles specific for Asian populations. As the taxonomic status between European and Asian beetles has been debated for years, mitochondrial as well as nuclear results support the recognition of *Ips subelongatus* as a valid species designation for Asian larch bark beetles.

KURZFASSUNG

Der Große Lärchenborkenkäfer *Ips cembrae* (Scolytinae) ist als Forstschädling an *Larix ssp*. im paläarktischen Raum zu finden. Diese Masterarbeit wurde durchgeführt, um Näheres über die Phylogeographie, Phylogenetik und Taxonomie von *I. cembrae* in Erfahrung zu bringen. Neun europäische und zwei asiatische Populationen wurden mittels mitochondrialer (*COI*) als auch nuklearer (ITS2) DNA Marker analysiert. Zusätzlich wurde überprüft, ob *I. cembrae* mit dem Endosymbionten *Wolbachia* infiziert sind, welcher die Zuverlässigkeit mitochondrialer Daten beeinflussen kann. Dazu wurde eine Standard-PCR mit spezifischen *wsp* Primern durchgeführt, die aber keinen Hinweis auf *Wolbachia* Infektionen lieferte. Weiters ermöglicht eine spezielle DNA Extraktion den Nachweis von nuklearen Kopien mitochondrialer Gene (NUMTs), die zu Artefakten in den abgeleiteten Stammbäumen führen können. Da anschließend an die Extraktion fehlerfreie DNA Sequenzchromatogramme generiert wurden, konnte ein Einfluss von NUMTs ausgeschlossen werden. Basierend auf den *COI* Daten teilte die phylogenetische Rekonstruktion die Haplotypen in zwei Kladen, wobei eine Klade die europäischen und die andere Klade die asiatischen Populationen gruppierte. *I. cembrae* aus Europa und Asien teilten keinen gemeinsamen Haplotypen. Innerhalb europäischer Populationen wurde eine geringe genetische Variabilität gefunden, während die Daten auf eine deutlich höhere Diversität innerhalb asiatischer Populationen hinwiesen. Ein großer genetischer Abstand wurde zwischen den europäischen und asiatischen Populationen festgestellt. Auch die Analyse der ITS2 Marker bestätigte eine klare Differenzierung zwischen europäischen und asiatischen Populationen, da zwei spezifische Allele ausschließlich in europäischen und zwei weitere Allele nur in asiatischen Populationen gefunden wurden. Die Ergebnisse der Studie stützen die Annahme, dass es sich bei dem asiatischen Lärchenborkenkäfer um eine eigenständige Art handelt, nämlich *Ips subelongatus.*

ACKNOWLEDGEMENTS

I would like to thank Christian Stauffer for his guidance and constant encouragement, allowing me to do my master thesis under his supervision in the insects genetics group. During the last months I have highly appreciated all the insightful and open-minded discussions we had. I am indebted to Coralie Bertheau for her abundant advice and high commitment in all respects. She provided timely and instructive comments at every stage of the thesis process. Due to her efforts we proved that a long distance is no obstacle for fruitful co-actions. Special gratitude should be given to Susanne Krumböck, whose expertise and good nature supported my performances in the lab. I especially thank Hannes Schuler and Peter Kern. They always made available their support in a number of ways and patiently answered all of my *Wolbachia* as well as non-*Wolbachia* related questions. Further I want to thank Ferenc Lakatos from the University of West Hungary, who provided precious time for guiding our beetles collection in the forests of Sopron. I enjoyed the excellent working climate and team spirit at the IFFF. Cordial thanks are due to all members for creating an outstanding setting for valuable discussions as well as for culinary adventures. I couldn't imagine a more pleasant companionship during my trip into the world of bark beetles.

Not at least I want to thank my family, whose unremitting support is with me in whatever I pursue.

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1. INTRODUCTION

1.1 Phylogeography, phylogenetics and molecular ecology

Reconstructing the evolutionary history of organisms and expressing it in form of a phylogenetic tree has always challenged scientists ever since the time of Charles Darwin (c.f. Häckel, 1866). As changes in morphological and physiological traits cannot draw a clear picture of evolutionary events, molecular analyses are expected to clarify many branching patterns of the tree of life which have been hard to solve by the classical approach. In this sense, phylogenetics plays an important role in developing a scientific basis of systematics, though it may not answer all the questions of the latter discipline (Nei & Kumar, 2000).

Phylogeography deals with the spatial and temporal dimensions of genealogy (Avise, 2009; Avise *et al.*, 1987) and emphasizes the principles and processes that shaped the contemporary geographic distribution of genealogical lineages, especially within and among closely related species (Beebee & Rowe, 2008). Avise and his colleagues (1987) aspired it as a bridge between population genetics and systematics, whereas microevolutionary processes (e.g. mutation, chromosomal change, etc.) operating within species can be extrapolated in order to explain macroevolutionary differences among species and higher taxa. Molecular techniques provide tools to study the historical events that established the present-day distribution of gene-based organismal traits, to trace kinship and to reconstruct migration patterns of populations and species.

In the course of replication and transmission of DNA from parents to the offspring, the hereditary script permanently undergoes mutations that alter molecular passages in the genomic sequence. Scientists have learnt how to read and to interpret the content of these evolutionary logbooks (Avise, 2006). Analyses of molecular processes, regulating the evolution of the genome, have been used to shed light on the history of given taxa (Carapelli *et al.*, 2008). However, as different regions of the genome can have different modes of evolution and transmission, they are affected differently by evolutionary forces in natural populations. Hence, investigations on various marker regions could translate into different consequences of mutation, drift, migration and

selection on patterns of geographical variation and molecular divergence (Navajas, 1998). From day one in the 1970s, animal mitochondrial DNA (mtDNA) sequences were the marker of choice for addressing precisely how conspecific individuals are genealogically linked through shared ancestors (Avise, 2009).

As Avise observed in 2009, phylogeography as a discipline 'has grown fantastically during the last 30 years and shows no signs of slowing down' (Avise, 2009). Thus, even this study makes use of phylogeographical principles and tools to unravel the interaction of genes in space and time. With this in mind, phylogeography is meant to explore the evolutionary relationship and structure of larch bark beetle populations, which are by now distributed throughout the world's largest ecozone, the Palaearctic.

1.1.1 Phylogeography of the Palaearctic biota

Investigations on historical backdrops that contributed to the current distribution and genetic variability of populations, species and communities, usually start with focusing on the most dominant palaeoclimatic feature, the ice ages. This approach has been proved highly informative, as surveys about the structure of numerous taxa have revealed patterns of genetic subdivision and diversity which are in line with isolation and separation in refugia during cold stages, and geographic and demographic re-expansion during the present interglacial (Hewitt, 2004).

2,5 million years before present (YBP) in the beginning of the Pleistocene, which is the first epoch of the Quarternary Period, the artic ice cap formed and the ice sheets in the northern hemisphere began to grow large. Quarternary climatic oscillations followed with temperature shifts of 7-14°C within only a few decades, inducing progressively larger ice sheets across Eurasia and North America. Especially the last 700 000 years have been dominated by major ice ages with a roughly 100 000 years-cycle, interrupted by relatively short warm interglacials such as nowadays (Hewitt, 1996). Approximately 18 000 years ago, during the last glacial maximum, the massive Laurentide ice sheet covered a huge part of North America as far south as 40°N, including the Great Lakes, while the Scandinavian ice sheet merely reached 52°N, covering parts of Great Britain and Northern Europe (Hewitt, 1996). During the major glaciations even the European mountain ranges of Cantabria, the Pyrenees, Alps,

Transylvania and Caucasus had considerable glaciation and the highlands of Scandinavia were completely glaciated (Hewitt, 1999 & 2000). Between the glaciated mountain blocks and the northern ice sheet, a plain of permafrost, tundra and cold steppe dominated which extended eastwards across Russia to the Urals (Hewitt, 1999) (Figure 1.1).

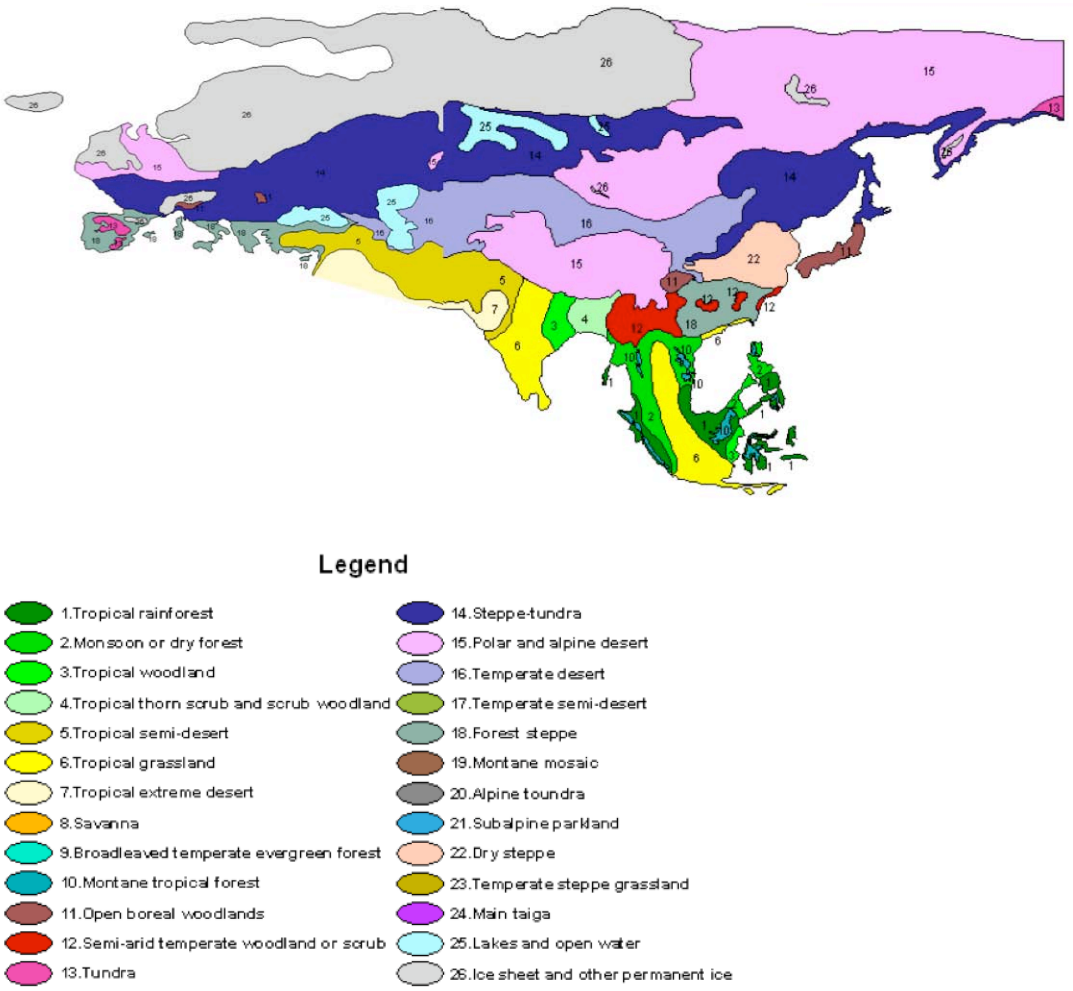

Figure 1.1: Vegetation map of Eurasia at the last glacial maximum 25 000–15 000 YBP. (Picture taken from Ray & Adams, 2001)

With each climatic oscillation flora and fauna went extinct, dispersed to new locations or separated into refugia. Biota migration was characterized by many cycles of contraction and expansion of geographical ranges. Therefore, contraction of ranges to regions south of the ice took place during cold periods, where suitable conditions

occurred for each species. When the ice retreated during subsequent warmings, species expanded their ranges from the leading edges northwards across areas of previously unlivable terrain (Hewitt, 1996). A well-studied network of pollen cores testified that species which now inhabit boreal and temperate regions in Europe, had their ice age refugia south of the ice and permafrost (Hewitt, 2000). There is good evidence that areas in the Carpathians, the present-day Moscow area, the Balkan, Italy and the Iberian Peninsula were the main refugial zones in Europe. Unlike Northern Europe, which was entirely covered by ice at the time of the last glacial maximum, northern Siberia and the Russian Far East were only locally glaciated (Bennett, 1997). Similarly, glaciation in Central and Northeast Asia was less extensive than in Europe, but biota strongly suffered from the cold and dry climate which dominated and caused the prevalence of tree-less landscapes and tundra communities (Edwards *et al.,* 2000). Thus, contemporary plant species near the European and Asian boarder probably survived in isolated refugia in Mongolia (Tarasov *et al.,* 2000), in the South of the Russian Far East (Korotky *et al.,* 1999) and in Southwest Beringia (Brubaker *et al.,* 2005).

From about 16 000 YBP the climate ameliorated for the longer term. Consequently, the ice sheets shrunk and species expanded their ranges out of the refugia northwards. Well-preserved beetle remains demonstrated that species with a contemporary Mediterranean distribution had reached Britain by this time (Atkinson *et al.,* 1987). At the end of the Pleistocene around 11 000 YBP, the rapid northern advance was sharply interrupted by another cold spell. But climate warmed again and by 6 000 YBP the vegetation pattern largely corresponded to that of nowadays. Bennett (1986) remarked that the rate of spread of plant species was notably rapid. Maps of radio dated pollen distribution from cores have shown that species expanded between 50 and 500 metres per year on average in Europe, pine and hazel moved even 1500m/year and alder 2000m/year (Bennett, 1986).

The postglacial migration patterns in the Palaearctic had great impact on the contemporary genetic structure of species. Each time when species spread after warming they colonized new territory, facing new environments and confronting new neighbours. These challenges would cause populations to diverge, both through selection and chance, and ultimately speciate (Hewitt, 2000). As some species would colonize from just one refugium and others from several, their genomes may mix to

various degrees, as it might have happened across the plains of Middle and Northern Europe (Hewitt, 1996). Reconstructing these demographic events is important for understanding the ecology and evolution of species, as e.g. extensive gene flow will constrain evolution by preventing local genetic differentiation, whereas reduced dispersal is expected to lead to spatial genetic subdivisions (Slatkin, 1987).

The range expansion out of refugia would presumptively be led by long-distance dispersants which set up colonies far ahead from the main distribution. Referred to as pioneer strategists (Hewitt, 1996), these long-distance dispersants were able to dominate the genome of the leading populations, whereas later migrants contributed little since they would be entering already established populations. Such a form of spreading from the leading edge was repeated many times over a long expansion route and probably involved a series of bottlenecks for the colonizing genome. Hence, these founding effects would lead to a loss of alleles, a tendency to homozygosity and low levels of diversity (Nei *et al.*, 1975). It is very likely that today's populations from northern parts of Europe, commonly possessing lower levels of diversity than southern populations, established via pioneer expansions (Horn *et al*., 2006). Hewitt (1996) specifies that slower and steady colonization was following the phalanx strategy. This expansion model tends to preserve most of the allelic diversity as several refugial genomes may spread and intermix more equally. As a result, the phalanx strategy causes less genome divergence among and within populations. It can be presumed that species in the southern ranges of Europe expanded this way, as populations survived by limited movements between suitable terrains (Horn *et al.*, 2006). Indeed, these changes of demographic ranges strongly corresponded to the local and regional topography of each region, such as size, latitude, orientation of mountains, lakes, valleys and plains (Hewitt, 1996). Recolonization in Europe was vastly impeded by mountains and seas running east-west which acted as major barriers to dispersal for many species and played a significant role in the historical subdivisions of populations. Those species surviving the ice age in refugia in Southern Spain, Italy, Greece or Turkey would have had the Pyrenees, Alps, Balkans and Caucasus to overcome when moving northward, those in North Africa had the Mediterranean Sea as barrier to cross (Hewitt, 1996). Taberlet *et al.* (1998) put forward the assumption of a general trend in European phylogeography, scilicet that the northern part of Europe was colonized primarily from the Iberic and the Balkan refugia, whilst populations evolving in Italian refugia were presumably not able to

spread northwards due to the Alpine barrier. A fact that yields in ‘southern richness and northern purity’ (Hewitt, 1999). Consequently, today’s distribution of many biota throughout the European continent is dissected by suture-zones which emerged when two postglacial colonization routes originating from distinct refugia made secondary contact, i.e. when populations with different or diverged genomes met (Taberlet *et al.*, 1998). The term suture-zone has been coined by Remington (1968) to describe ‘a band, whether narrow or broad, of geographical overlap between major biotic assemblages, including some pairs of species or semispecies which hybridize in the zone’. Four main suture-zones have been detected in European biota (Taberlet *et al.*, 1998), usually located near natural barriers or in areas where lineages expanding from different refugia merge (Figure 1.2).

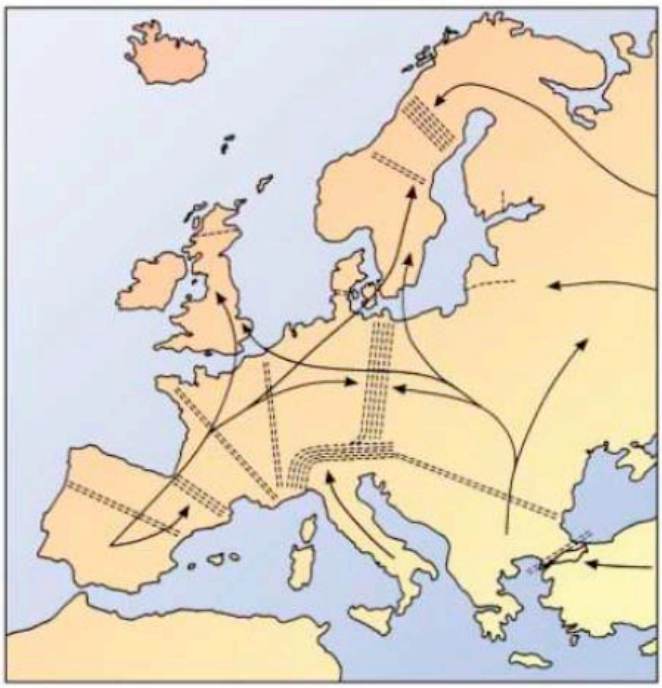

Figure 1.2: The general position of well known hybrid- and suturezones in Europe: arrows correspond to glacial refugia, direction and length of postglacial expansions; geographic barrieres are illustrated by dotted areas. Major clustering is indicated in Scandinavia, Central Europe and the Alps. Other clusters are apparent in the Pyrenees and the Balkans. These suture zones are caused by commonalities of ice age refugia, rate of postglacial expansion and physical barriers. There is further subdivision in the southern regions. (Picture taken from Hewitt, 2000)

Taberlet *et al.* (1998) concluded that the contribution of potential easternmost refugia, localized in Europe and/or Asia, might be underestimated for reason of insufficient data about Eastern Europe and Fennoscandia. Thereupon a recent publication indicated that the area east of the bounder of the Scandinavian ice sheet to the Ural Mountains held isolated patches of trees during the late-glacial periods and in the early Holocene (Väliranta *et al.*, 2011). These small populations acted as initial nuclei for population expansion and forest development in northeastern European Russia (Väliranta *et al.*, 2011). However, it is assumed that the formerly glaciated Verchoyansky Mountains on the eastern shore of the Lena River acted as a strong barrier to gene flow throughout this region. But as to say, the postglacial recolonization of Russia and northern Siberia by biota is still poorly understood (Semerikov, 1999).

The impact of quaternary glaciations in Asian local biota is a topic of considerable interest, as the Sino-Japanese Floristic Region harbours the world's most diverse temperate flora (Qui *et al.*, 2011). Presumably this region acted as an important glacial refugium throughout Quaternary ice age cycles in Central and East Asia. During the last glacial maximum many smaller mountain glaciers formed in Southeast Asia and permafrost covered Asia down to Beijing. A recent study puts forward that during the late Pleistocene an increase in the amount of climatically mild habitats in East Asia occurred, due to a decline in elevation and the development of monsoons since the Mid-End Pleistocene (Ding *et al.*, 2011). A steadily increasing number of phylogeographic studies in the Sino-Japanese Floristic Region of mainland China (and adjacent areas including the Qinghai-Tibetan-Plateau and Sino-Himalayan region) has documented the population histories of temperate plant species in these regions. A review of current literature reveals three phylogeographic scenarios, which have been listed by Qui *et al.* (2011). Firstly, the postglacial recolonization of the Qinghai-Tibet-Plateau started from (south-) eastern glacial refugia. Furthermore, the population isolation and endemic species formation in southwestern China established due to tectonic shifts and river course dynamics. And thirdly, a long-term isolation and species survival in multiple localized refugia of (warm-) temperate deciduous forest habitats in subtropical (Central/East/South) China must have occurred (Qui *et al.*, 2011).

As to summarize, the European postglacial recolonization has been comparatively well studied, whereas the demographic history in Asia is quite unexplored. Nonetheless, having a rough idea of ice age refugia and postglacial recolonization routes may help reconstructing processes which had major effects on the contemporary genetic and spatial structure of species.

1.2 Biology and ecology of the eight spined larch bark beetle *Ips cembrae*

Ips cembrae (Heer 1836) is a blackish brown, 4-6 mm sized insect belonging to the subfamily Scolytinae (Coleoptera) (Marvaldi & Morrone, 2000). *I. cembrae* cannot easily be distinguished from its close spruce relatives *Ips typographus* and *Ips amitinus* by morphological traits, as each of them has four equally spaced spines on each side of the elytral declivity. However, the declivity area of *I. cembrae* is glossy, whereas *I. typographus'* is matt. The third spine of *I. cembrae* is the largest spine and strongly capitate. Moreover, the surface of the elytral depression is covered with long, yellowish hairs (Wood & Bright, 1992; Pfeffer, 1995) (Figure 1.3). Zhang & Niemeyer (1992) pointed out that a combination of second and third elytral spine characteristics allows separating both *I. cembrae* sexes with accuracies of 99% (males) and 97% (females), respectively.

Figure 1.3: Adult beetles of the eight spined larch bark beetle *Ips cembrae*.
(Picture taken from the Pest and Diseases Image Library's Images: www.forestryimages.org/browse/detail.cfm?imgnum=5325074)

I. cembrae is a polyphagous secondary pest on various *Larix* spp. throughout Europe and Asia (Schimitschek, 1930; Sawamoto, 1940; Nobuchi, 1974; Postner, 1974; Pfeffer, 1995). Trees of the genera *Abies*, *Pinus* and *Picea* may occassionally serve as hosts, too. In larch forests of the Alps and Carpathians in Central and Southeast Europe the beetle is an indigenious species with *Larix decidua* as its main host. In general, *I. cembrae* does not present a particular risk and is an economically less important forest pest than e.g. *I. typographus* (EPPO/CABI, 1997). However, *I. cembrae* has also spread to northern *Larix* forests and non-native *Larix* plantations (e.g. *Larix leptolepis*), such as to the Netherlands, to parts of Great Britain and Scandinavia (Redfern *et al.*, 1987; EPPO, 2005), probably as a result of larch lumber trade. Within the last years many conifer plantations have been established, including *L. decidua* and other *Larix* spp. In terms of phytosanitary control it is desirable to maintain these zones free from other European and non-European bark beetles (EPPO, 2005).

Primarily, *I. cembrae* infests weakened or wind thrown trees and harvested trunks. Thus, Bevan (1987) reports that an *I. cembrae* population, introduced in the United Kingdom, was able to invade relatively healthy trees suffering from drought stress. High *I. cembrae* population levels that follow after thinning and logging are meant to favour these infestations, too. Moreover, at high population densities *I. cembrae* may also attack healthy trees and cause long-lasting and destructive outbreaks (Zhang & Niemeyer, 1992).

Generally, adults leave their host tree and hibernate in the forest litter whereas pupae, larvae and some imagines hibernate under larch bark. The swarming season of the parent generation takes place on warm days in late April and early May, depending on altitude and geographic site. Males infest host trees, excavate nuptial chambers and attract females by pheromones. After mating in the nuptial chamber females bore mother galleries in the phloem-cambium inner bark layer with a variabel length of up to 25 cm, carving the wood. Eggs are deposited in egg nieches, where they develop for about one to two weeks. Subsequently, newly hatched larvae start constructing larval feed galleries. After passing larval and pupae stages young imagines emerge from the tree and have a maturation feed either in the branches of younger trees or near to the brood gallery, given that fresh bark is still present. Altogether it takes

about nine weeks after egg deposition that young imagines start swarming (Nierhaus-Wunderwald, 1995). Depending on the length of the summerseason, one or two annual filial generations may occur next to sister broods. Adults need additional regeneration feeding, which usually occurs on the trunk near the region of larval development but may sporadically be on roots and at the zone of thin bark at the top of the trunk and on the branches (Zhang & Niemeyer, 1992). At worst, larvae and adult beetles may encircle trunks by feeding in the bark and disrupting the water and assimilate transport between roots and crown. Besides, maturation and regeneration feeding of adults cause much damage in crowns because of nibbled shoots which can easily be broken by wind (Nierhaus-Wunderwald, 1995).

I. cembrae individuals have an average life expectancy of about a year. Experiments have shown that *Ips* ssp. adults can fly continuously for several hours up to a few kilometres, normally downwind (EPPO, 2005). Direct observations of *I. typographus* revealed migration distances up to 30 km (Gries, 1985). Dispersal over longer distances mostly depends on transportation under the bark of logs.

Ips ssp. are known to be associated with blue-stain fungi belonging to the genera *Ophistioma* and *Ceratocystis* (Wingfield *et al.*, 1993; Paine *et al.*, 1997; Kirisits, 2004), which often implicate timber devaluation. Both organisms gain benefit, as the fungi are transported to new host trees by the beetles and support them to overcome the host trees defense response. In particular, *I. cembrae* is known to act as a vector of blue-stain fungus *Ceratocystis laricicola* (Redfern *et al.*, 1987; Kirisits, 2004).

1.2.1 The Asian larch bark beetle *Ips subelongatus*

The taxonomy of *Ips* species attacking *Larix* ssp. in Europe and Asia is still unclear. Separated on basis of their host plants, geographical distribution or morphological similarities, several authors (Wood & Bright, 1992; Pfeffer, 1995 c.f.) have considered *Ips fallax*, *Ips shinanonensis* and *Ips cembrae* var. *engadinensis* as synonymous designations of *Ips cembrae*. The Asian larch bark beetle has been considered as *Ips subelongatus* by Russian and Chinese entomologists, whereas European colleagues treated this species name as a synonym of *I. cembrae* (Zhang *et*

al., 2007). According to Yin *et al.* (1984) as well as to He *et al.* (1988), only *I. subelongatus* is morphologically distinct from *I. cembrae*. He *et al.* (1988) found two types of second spine shape distributed to the sexes in proportions completely different from those in European *I. cembrae*. Other references claim that there are no differences in morphological traits (e.g. Wood & Bright, 1992; EPPO, 2005). Therefore, *I. subelongatus* can be regarded as a sibling species of *I. cembrae*.

A phylogeographic study conducted by Stauffer *et al.* (2001) investigated the relationship of Palaearctic eight spined larch bark beetles. The results suggested that the *I. cembrae* complex includes at least two taxa, i.e. *I. cembrae* in Europe and *I. subelongatus* in Asia (see chapter 1.2.2). As highlighted by the comprehensive phylogenetic study on *Ips* species by Coganto & Sun (2007), *I. subelongatus* should be considered as a valid species designation for the Asian larch bark beetle (Figure 1.4).

I. subelongatus is distributed throughout Central Asia, such as China, Japan, Mongolia, Russia and the Republic of Korea, but also in the northeast of European Russia and Sibiria (Pfeffer, 1995). The beetle mainly infests *Larix sibirica*, *Larix kaempferi*, *Larix olgensis* and *Larix gmelinii* (Schimitschek, 1930; Nobuchi, 1974; Postner, 1974). *I. subelongatus* is also known to act as a vector for a blue-stain fungus, which may damage the tree or devaluate timber (Stauffer *et al*., 2001). Marin *et al.* (2005) investigated the fungi associated with European and Asian larch bark beetles. The findings showed that the fungus associated with the Asian beetles was morphologically the same as the fungus associated with the European beetles. However, genetically it represented a discrete taxon that is closely related but nevertheless dinstinct from the European fungus *Ceratocystis laricicola*. The Asian fungus was then described as *Ceratocystis fujiensis* (Marin *et al.* 2005).

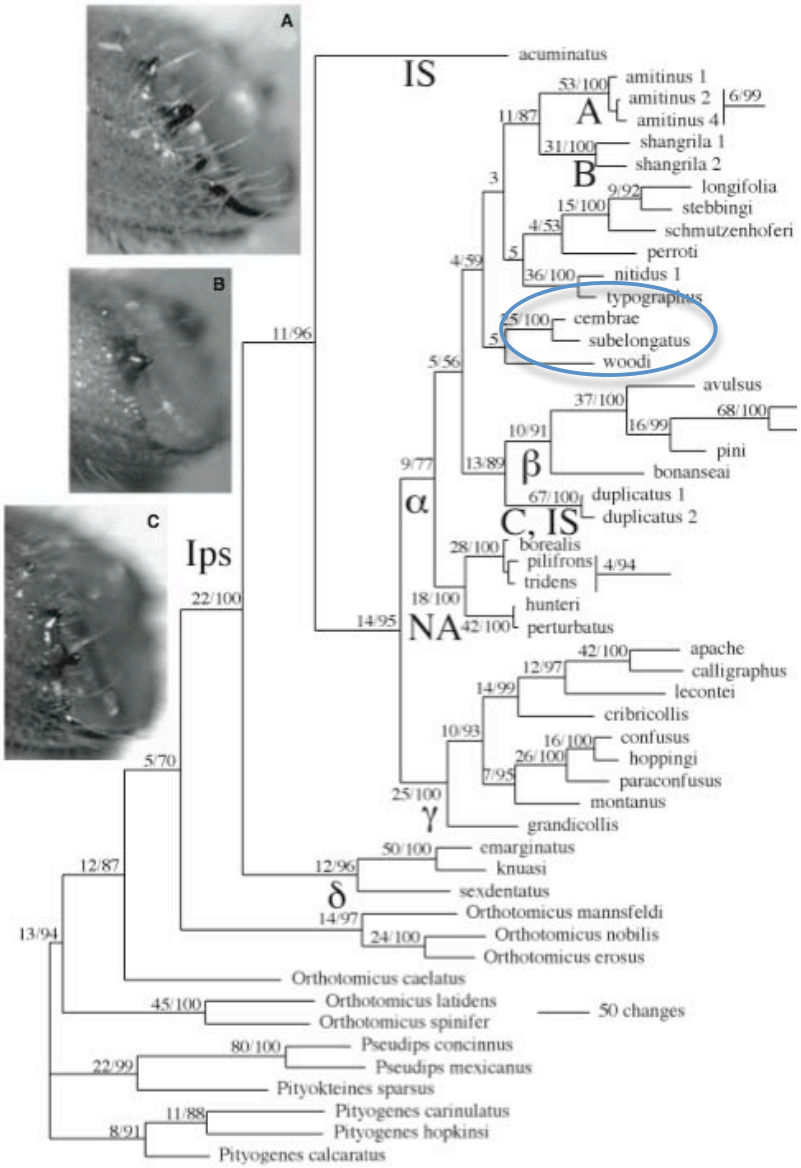

Figure 1.4: Maximum Parsimony tree of all 23 Nearctic and 12 out of 13 Palaearctic *Ips* species, revealing *Ips cembrae* and *Ips subelongatus* as own taxonomic entities. (Picture taken from Cognato & Sun, 2007)

1.2.2 Former studies of *Ips cembrae* and *Ips subelongatus*

Ips cembrae is just one out of 36 species related to the genus *Ips* De Geer. Distributed throughout conifer forests of the northern hemisphere, these species use the phloem-cambium inner bark layer as food, for breeding and for nurturing the offspring (Wood & Bright, 1992). 23 out of these 36 *Ips* species occur merely in the Nearctic region throughout North and Central America and the remaining 13 in the Palaearctic region of Eurasia, whereas it is noted that seven *Ips* species are native in Europe (Wood & Bright, 1992). Cognato & Sun (2007) criticize that especially the Asian *Ips* species diversity is poorly studied. It seems widely accepted that species diversity in Asia is likely much higher than currently known, and even underestimated for reasons of inaccessible, remote areas and a lack of local scolytid taxonomic expertise. Several publications indicate that a more diverse Asian *Ips* fauna has to be taken into account (Stauffer *et al.*, 1999; Cognato & Vogler, 2001; Cognato & Sun, 2007).

Several studies exist about postglacial recolonization of the most important forest pest *Ips typographus* and its host in Europe (e.g. Lagercrantz & Ryman, 1990; Schmidt-Vogt, 1991; Sallé *et al.*, 2007; Tollefsrud *et al.*, 2008 & 2009). Data are contradictory as mitochondrial analyses on the insect showed a migration pattern from south to north and no influence from the Russian spruce refugial area to Central Europe, whereas nuclear data based on microsatellites revealed very high gene flow but no interpretable pattern (Sallé *et al.*, 2007; Stauffer *et al.*, 1999). Few studies exist on the postglacial history and structuring of European *Ips cembrae*. Stauffer *et al.* (2001) analyzed European and Asian larch bark beetles and formed two significantly distinct genetic clusters, based on the mitochondrial DNA. The degree of 4,3% differentiation observed among populations of *I. cembrae* suggested the recognition of Asian *Ips subelongatus* as an own taxonomic entity. The study indicated that European *I. cembrae* survived glaciation next to its main host, as larch was mainly restricted to the Alps (Stauffer *et al.*, 2001). Three haplotypes were detected in European populations whereby haplotype I and II were found close to the glacial refugial areas in Poland and the southwestern Alps (Figure 1.5). Surprisingly, haplotype III occurred only in an allochthonous *Larix* stand in Scotland, where the insect was recently introduced by timber trade (Redfern *et al.*, 1987). These data contrasted the common hypotheses that fewer haplotypes are found in regions of recent introduction (Hewitt, 1996). Thus

Stauffer *et al.* (2001) assumed that haplotype III occurred in Europe as well, but was not found due to the small sample size. Moreover, the study revealed two haplotypes (IV-V) in Asia, whereby haplotypes I-III and IV-V differed in 20 nucleotide sites. Data analyses showed that haplotype V (Central Asia) evolved either from haplotype II or haplotype IV (Japanese population). The divergence in the mt*COI* gene revealed that the European and Asian larch bark beetles diverged during the Pleistocene. According to Stauffer *et al.* (2001), this speciation event proceeded by geographic isolation rather than by sympatric host adaptation.

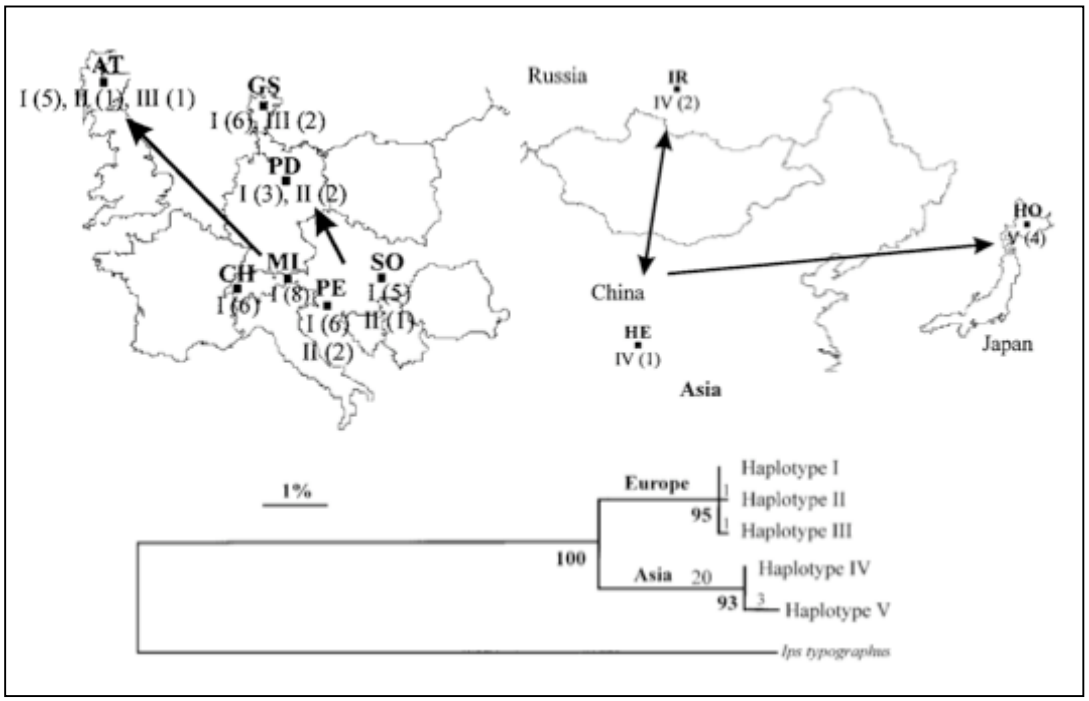

Figure 1.5: Distribution of haplotypes I, II, III (Europe) and IV, V (Asia); Maximum parsimony tree as calculated by Stauffer *et al.* (2001).

A recent study investigated the electrophysiological and behavioural responses of *Ips subelongatus* to semiochemicals from host, non-hosts and conspecifics in China (Zhang *et al.*, 2007). A striking result of this investigation was that *I. cembrae* and *I. subelongatus* responded differently to pheromone components. Field trapping experiments demonstrated that pheromone components of European larch bark beetles, namely ipsdienol and 3-methyl-3-buten-1-ol, were unattractive for both sexes

of Asian larch bark beetles. By implication the authors added support to the phylogenetic finding of Stauffer *et al.* (2001), namely that European and Asian larch bark beetles are best treated as two distinct species: *I. cembrae* infesting larch in Europe and *I. subelongatus* infesting larch in Asia.

1.2.3 Phylogeography of *Larix* ssp.

Larch species are important components of boreal and temperate forests. They are widely distributed across Eurasia and constitute up to 40% of its tree-covered regions (Farjon, 1990). *Larix decidua* and *Larix sibirica* are the only larch species native in Europe (Kral, 1979; Huntley & Birks, 1983). The former is distributed along the Alps, southern Poland, throughout the Tatra region, lower ranges of the Carpathians and the Roman Carpathians. *L. sibirica* is regarded as a western segregate of the widespread Siberian Larch and occurs in northwestern Russia (Huntley & Birks, 1983). Besides its natural habitat, *L. decidua* has been widely planted throughout Europe during the last four centuries. Furthermore, its near relative *Larix kaempferi*, the Asian-native Japanese larch, and the hybrid between these two species, *Larix x eurolepis*, have been introduced to Europe and planted mainly for timber production.

Compared to the main tree species in European forestry, *Picea abies*, little is known about the late glacial and postglacial history of larch. *L. decidua* may have arisen in the European mountains since the late Pleistocene and could have evolved through geographical isolation and speciation from an extinct Eurasian taxon (Huntley & Birks, 1983). *L. decidua* was consistently present in a refugium in Poland during the last two interglacials (Kral, 1979). During the late glacial period (13000 - 10 000 YBP) larch occurred on the south side of the Alps and in the Polish Carpathians from where a northward expansion across the Polish lowlands took place. Fossil pollen maps displayed that by 7000 YBP larch declined and apparently disappeared in Poland during the early Holocene. Subsequently, larch was restricted to the Alps although present for the first time in the Alpes Maritimes. At that time larch was likely bound to the highest altitudes due to competition with other more thermophilous taxa, e.g. European beech *Fagus sylvatica*. The higher ascent of these

latter taxa at 7000 YBP suggests a climate more favourable than today's, but some cooling deterioration was already established by 6000 YBP (Huntley & Birks, 1983).

There is little evidence for large-scale migration of larch in Poland. The early Holocene decline is most easily explicable as a restriction to a few scattered montane localities, caused by climatic amelioration and the invasion of thermophilous trees. However, this explanation ignores two important factors. Firstly, larch is found not only in the highest Carpathian ranges of Poland today but also in the much lower mountains of the Little Poland Highland. Secondly, a separate taxon of larch is recognised in Poland next to typical *L. decidua*, namely *L. decidua* ssp. *polonica*. Today *L. decidua* survives in Poland only in the High Tatra, just as it persists in scattered localities elsewhere away from the main Alpine ranges. These scattered localitites may result from long-distance transport from the Alps during the Holocene as *L. decidua* had small refugia in these mountains during the last glaciation. These two possibilities cannot be evaluated at present as the localities are small and detailed pollen analyses are lacking. However, the failure of *L. decidua* to migrate further north from northern Poland is puzzling. Equally unexplained is the exclusion of *L. decidua* ssp. polonica from high montane forests in Poland, coupled with its replacement in the highest ranges by *L. decidua*. The contrast between these two taxa may reflect differentiation under glacial and interglacial conditions, respectively. *L. decidua* ssp. *polonica* has possibly been adapted to low-altituded boreal forest environments experienced during glacial stages, whereas *L. decidua* was distributed to high-elevated montane forest environment experienced during temperate stages, but restricted by the presence of glaciers in the mountains during glacial stages (Huntley & Birks, 1983).

Along with recent phylogeographic findings provided by macroscopic plant remains, it has become evident that the current assumptions of tree migration need re-evaluation. Newly presented data from Western Europe show that arboreal vegetation had glacial refugia much further north and that trees existed during the late-glacial closer to the ice margin than previously thought (Väliranta *et al.*, 2011). For a long time, the vast forest and tundra area in northeastern European Russia, east of the Scandinavian ice sheet, has been a poorly investigated region in terms of studies about post-glacial tree colonization and forest development. Lately Väliranta *et al.*

(2011) indicated that the post-glacial genesis of forests in non-glaciated areas occurred through expansion in the size and density of isolated tree population patches rather than through a broad-scale northwards and thus Central Europe like movement from distant southern refugia.

Open landscapes dominated during the last glacial maximum 18 000 YBP in Beringia and Northeast Asia (Edwards *et al.*, 2000). As elucidated in chapter 1.1.1, recent data support the assumption that refugia harbouring tree species were located in western Beringia next to a refugium along the drained seashore of Okhotsk Sea. There is good evidence that tree species such as *Larix, Betula, Populus* and others survived glaciation in this region (Tarasov *et al.*, 2007; Brubaker *et al.*, 2005). Next to that, Semerikov *et al.* (2007) and Khatab *et al.* (2008) suggested that the current distribution and genetic variation of *L. sibirica* and *L. gmelinii* could reflect Pleistocene extinction and postglacial recolonization of the northern part of the taiga zone from southern glacial refugia.

1.3 Molecular approaches in phylogeography and phylogenetics

1.3.1 The mitochondrial genome

In the late 1970s the discovery of mitochondrial DNA (mtDNA) as a useful molecular marker has rapidly enhanced evolutionary analyses (Zhang & Hewitt, 1996 & 2003; Behura, 2006; Avise, 2009). MtDNA is still the most popular genetic marker used in molecular ecology, i.e. for phylogeographic purposes, and has been applied in more than 70% of scientific papers (Avise, 2000). However, its supremacy has decreased in recent years due to alternatives as microsatellites and other nuclear multicopy markers. In relation to this, Behura (2006) emphasized that the choice of applying a specific marker system depends upon the objectives of the study, and mtDNA still retains several advantages for phylogeographic investigations.

According to one version of the endosymbiont hypothesis, mitochondria are thought to be evolutionary remnants and originated from free-living Alpha-proteobacteria that were integrated by early ancestors of today's eukaryotic cells (Campbell & Reece,

2006). A cell contains many mitochondria which may occupy up to 25% of its cytoplasm volume (Ballard & Whitlock, 2004). The mitochondrial genome is an extra-nuclear, circular double-stranded molecule that ranges in size from 16 000 to 20 000 nucleotide pairs (Avise, 2006). Mitochondria include multiple copies of their own circular DNA genomes which consist of 37 functional genes (Figure 1.6). 22 tRNAs and two rRNAs encode the translational machinery of the mtDNA itself, whereas 13 genes are protein-encoding (Ballard & Whitlock, 2004). Six out of these 13 proteins are involved in the oxidative phosphorylation of the respiratory chain including subunits I-III of cytochrome *c* oxidase (*COI-COIII*) (Beebee & Rowe, 2008).

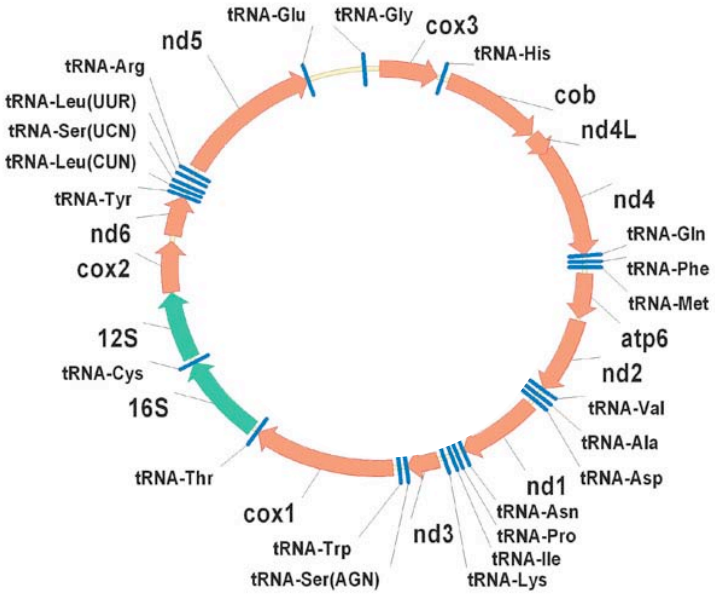

Figure. 1.6: The animal mitochondrial genome (Picture taken from Cai *et al.*, 2011).

Animal mtDNA was thought to be inherited maternally because sperm mitochondria are destroyed after fertilization. Just a few exceptions of biparental inheritance were reported so far (Ballard & Whitlock, 2004; Beebee & Rowe, 2008). However, the usually assumed uniparental transmission highlights a favorable property for phylogenetic analyses, as mtDNA does not undergo recombination, possesses haploid status and only one locus. Hence, mtDNA mutations arising in different individuals are not recombined during sexual-reproduction and evolution at the nucleotide

sequence level is rapid (Avise *et al.*, 1987). This fact led to a widely applied clock calibration for animal mtDNA: an about 2% sequence evolution between pairs of lineages per million years is estimated, which means 1% sequence evolution per lineage per 106 years (Avise, 2000).

The generally high mutation rate of mtDNA results in an up to 10 times higher genetic variation compared to single-copy fractions of the nuclear genome (Avise *et al.*, 1987; Behura, 2006). Several hypotheses have been put forward to explain this fast accumulation of nucleotide divergence in animal mtDNA (Richter, 1992; Li, 1997; Nebdal & Flynn, 1998). One explanation might refer to the relaxation of functional constraint as mtDNA does not code for proteins directly involved in its own replication or transcription. Furthermore, the mitochondrial molecule produces only 13 kinds of proteins and might tolerate less accuracy in translation (Duran, 2003). The high mutation rate could also origin in an inefficient DNA repair mechanism, which expectably causes higher polymorphism. In addition, this intraspecific nucleotide polymorphism in mtDNA is considered to be effectively neutral. Put simply, the distribution of haplotypes, i.e. mitochondrial genotypes, must be influenced by demographic events rather than by selection (Beebee & Rowe, 2008). The mutational dynamics of mtDNA sequences, uniparental inherited and thus in the absence of recombination, facilitate the investigation on genetic relationships among haplotypes. As the effective population size of mtDNA is one quarter that of diploid nuclear genes, haplotype frequencies can drift rapidly, creating genetic differences among populations in relatively short time (Beebee & Rowe, 2008). Another issue worth mentioning is that the mitochondrial genome of animals is highly compact and does not contain introns or long non-coding spacer sequences that character nuclear genomes (Avise *et al.*, 1987). Moreover, versatile PCR primers, often designed from highly conserved mitochondrial genes as *COI*, enable simple and conventional amplifications and allow successful PCR even from museum material or archaeological remains (Beebee & Rowe, 2008).

In spite of its usefulness in phylogeography and phylogeny, mitochondrial data are associated with a number of pitfalls which may question and bias the reliability of mtDNA based studies (Zhang and Hewitt, 1996). Because it is inherited only through the maternal line, the analysis of mtDNA often gives a wrong and incomplete picture,

in particular when males and females differ in disperal patterns. Although the information recorded in mtDNA represents only one of many molecular tracings in the evolutionary histories of organisms, it is nonetheless a specified genealogical history, namely that of the females (Avise *et al.*, 1987).

Compared to nuclear autosomal sequences, mtDNA lineages have a much faster lineage-sorting rate and higher allele extinction rate. Analyses may result in an oversimplification of evolutionary relationships, underestimated genetic diversity, increased uncertainty concerning correctness of haplotypes and incorrectly detected remote population processes (Zhang & Hewitt, 2003). Due to the unilateral mode of inheritance and paternal leakage, an individual is commonly assumed to carry only one mtDNA haplotype (Ballard & Whitlock, 2004). Each cell has hundreds of mitochondria, each containing 2 to 10 copies of mtDNA molecules. In one specific cell mtDNA are usually identical, a condition known as homoplasmy. Recently, studies have shown that individuals can be heteroplasmic, carrying more than one unique mitochondrial genome (White *et al*., 2008). Heteroplasmy may complicate the assignment of a single diagnostic and individually orthologous mtDNA haplotype (Berthier *et al.*, 2011). Individuals with heteroplasmic mitochondrial genomes could result from incomplete elimination of mutant mitochondrial lineages within a cell or tissue, subsequent heterologous recombination, paternal mtDNA transmission or hybridizations (White *et al*., 2008).

Further on, studies have referred to another common source of intra-individual variation, namely the incorporation of mitochondrial fragments into the nuclear genome (NUMTs) (Zhang & Hewitt, 1996; Bertheau *et al.*, 2011). This phenomenon of nuclear pseudogenes of mitochondrial origin needs better understanding in order to avoid misleading data analyses, as PCR may amplify these insertions. Mistakenly a NUMT could then be interpreted as a true mitochondrial gene (Ballard & Whitlock, 2004). Care must be taken since especially cryptic NUMTs, possibly differing by only 1-3 bp from authentic mitochondrial haplotypes, might be responsible for haplotype richness found in several species, for instance *Ips typographus* (Bertheau *et al.*, 2011).

Maternally inherited endosymbionts like *Wolbachia* may also hinder correct analyses of mitochondrial data. *Wolbachia* are intracellular bacteria which are found in arthropods and nematodes. A recent meta-analysis estimated that more than 65% of insect species might harbour *Wolbachia* (Werren *et al*., 2008). However, conservative estimations based on standard PCR detection suggested, that 16 to 22% of insect

species are infected with *Wolbachia* (Werren & Windsor, 2000). The endosymbiont alters the hosts biology and induces reproductive manipulations, such as feminization, parthenogenesis, male-killing and cytoplasmatic incompatibility (Werren *et al.*, 2008). Therefore, the presence of *Wolbachia* is known to influence the mitogenome by causing a reduction of its hosts mtDNA polymorphism (Turelli & Hoffmann, 1991).

1.3.2 The nuclear genome

The nuclear genome of insects encodes several thousands of different proteins and exists in two copies per diploid cell (Beebee & Rowe, 2008). The vast majority of the nuclear genome accounts for non-coding sequences like introns or untranslated intergenic regions, also named junk DNA, whereas protein-coding genes form the minor part of it. These structural genes are often under strong natural selection. Therefore, the variation among individuals in the sequences of these proteins is often too low to be appropriate for phylogenetic analyses (Beebee & Rowe, 2008). Nonetheless, some variability exists and has mainly been studied at the protein rather than the DNA level. Nowadays nuclear DNA (nuDNA) sequence alterations, so-called ‚single nucleotide polymorphisms' (SNPs), can be detected more easily (Beebee & Rowe, 2008) and thus facilitate and promote the application of nuclear markers (Zhang & Hewitt, 2003).

Nuclear markers inherit characteristics which allow to overcome the limitations of mtDNA. Due to the biparental inheritance mode, nuclear DNA undergoes permanent recombination and favours tracing back the demographic history of the matrilineal and patrilineal history, bygone hybridisation or gene flow. Analysis is complicated by the fact that, apart from nucleotide substitutions, insertions/deletions (indels) often constitute a large part of the polymorphism detected (Zhang & Hewitt, 2003). Indels are usually either ignored or treated as ambiguities, although the use of information contained in these genome regions is important for proper interpretations and explorations of polymorphism. Furthermore, individuals can be heterozygous at a given locus (i.e. possess two different forms of a gene). Separating these alleles for

detecting an allele-specific characterization is a very time-consuming, expensive and technically challenging analytical procedure (Zhang & Hewitt, 2003).

Along the nuclear genome the mutation rate and therefore the distribution of polymorphic sites differ greatly. Especially the rRNA genes have long been recognized as attractive markers for phylogenetic studies (Hillis & Dixon, 1991). These genes are organized in clusters of repeated units, each of which consists of coding sequences and several transcribed and non-transcribed spacer regions (NTS). The transcription units include the 18S, 5.8S. and 28S genes as well as external and internal transcribed spacers (ETS and ITS). For instance 18S-rDNA and coding single-copy ncDNA are quite conserved, while other loci like introns, the ribosomal internal transcribed spacer (ITS) or mini- or microsatellites, evolve at much faster rates (Arthofer, 2005). Therefore, ITS marker appear to evolve 2,5 times faster than the mitochondrial *COI* region (Navajas, 1998). Hence, the rDNA clusters have the potential to reveal phylogenetic relationships at many taxonomic levels (Zhang *et al.*, 2004). Importantly ITS2, which has proven to be an excellent nuclear marker for similarly aged radiations in organisms like fungi and plants, is only rarely used for phylogeny estimation in arthropods, although universal primers exist. This is partly due to difficulties in the alignment of ITS2 sequences in more distant taxa (Wiemers *et al.*, 2009). Phylogenetic information can also be revealed from other nuclear regions such as rRNA loci, regulatory domains flanking structural genes or particular subsets of non-coding sequences (microsatellites), which are often highly repetitive. Microsatellites are short tandem repeats made up of 1-6 nucleotide motifs. Proprietary mutational mechanisms cause high substitution rate and polymorphism at such loci. Locus specific primers must be developed de novo for each species to be analyzed for the first time (Arthofer, 2005). In such a way, attempts to isolate microsatellites during a study on the phylogeography of the six toothed bark beetle *Pityogenes chalcographus* were unsuccessful (Arthofer *et al*., 2007). Several publications report that scolytids not only have few microsatellite loci, but also that those which have been isolated seem to be less polymorphic than those in other insect species (Stauffer, 2004; Arthofer *et al*., 2007; Sallé *et al.*, 2007). Versatile primers like those available for mitochondrial genes are not found yet, or do not satisfy scientific requirements (Zhang & Hewitt, 2003).

2. RESEARCH OBJECTIVES

This master thesis was designed to perform a phylogeographic and phylogenetic investigation on European and Asian *Ips cembrae* populations. Inter- and intraspecific analyses can provide novel indications of divergence between and among these two geographically separated entities. Thus the aims of the thesis can be summarized as follows:

1. Clarification on possible pitfalls analyzing the mtDNA of *I. cembrae* populations:
    - Is the bacterial endosymbiont *Wolbachia* present in *I. cembrae* and if, does this endosymbiont influence the mitochondrial genetic structure?
    - Are NUMTs present in *I. cembrae* and if, do these pseudogenes influence the mitochondrial dataset?

2. Phylogeography of European and Asian larch bark beetle populations:
    - What is the genetic structure of European and Asian populations and is there any gene flow among these two geographically separated entities?
    - Do examinations of both mitochondrial and nuclear markers show the same outcomes or do they differ in phylogeographic results ?
    - What conclusions can be drawn on the postglacial history of European and Asian *I. cembrae* populations?

3. The genetic relationship between European and Asian larch bark beetles:
    - Is *Ips subelongatus* an appropriate and taxonomically justified designation for Asian populations?

3. MATERIALS AND METHODS

3.1 Location of collections

Ips cembrae specimens were from the collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection, Boku, Vienna. The European and Asian populations were collected in 1996/1997 and 2001, respectively. The population from Hungary, specifically used for NUMTs analyses, was sampled in May 2011. Asian populations were located in northeastern China and Inner Mongolia, respectively. As mitochondria are inherited matrilineal, only one individual per mother gallery was collected, in order to avoid biased haplotype diversity. All specimens were stored in 96% ethanol at -20°C. Sample localities are shown in figure 3.1. A complete overview of sampling sites and number of specimens investigated is given in table 3.1. Overall, 11 populations were investigated with 152 individuals used for mitochondrial DNA analyses and 55 individuals for nuclear DNA analyses.

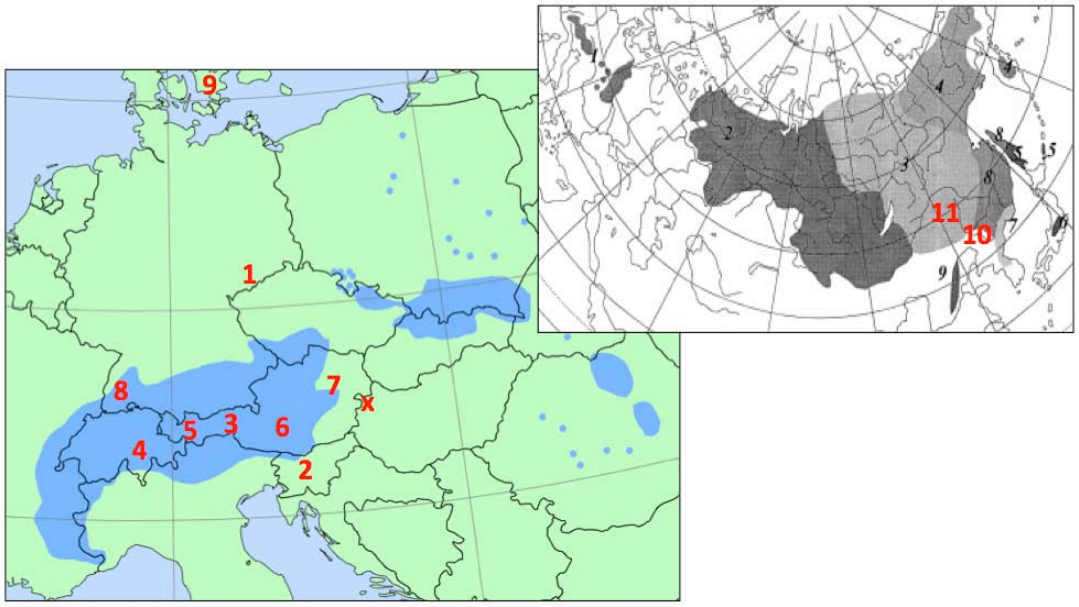

Figure 3.1: Sample sites of and Asian *I. cembrae* populations and distribution of *Larix* ssp.: Populations are numbered 1-11 and x (Hungarian population). Left map: Distribution of *Larix decidua* is shown in blue. Right map: Distribution of *Larix* ssp. in Eurasia. Numbers in black correspond to taxa: *1 L. decidua, 2 L. sibirica. 3 L. gmelinii. 6 L. kaempferi. 7 L. olgensis. 8 putative hybrid L. gmelinii L. olgensis, 9 L. gmelinii var. principis rupprechtii.*

Table 3.1: Numbers of populations, collection sites, date of capture and numbers of specimens analyzed for *COI*, ITS2, *Wolbachia* and NUMTs.

N°	Locality	Date of capture	*COI*	*ITS2*	*Wolbachia*	NUMTs
Pop1	GERMANY/Tharandt	1996/1997	15	5	-	-
Pop2	SLOVENIA/Martuljek	1996/1997	15	5	-	-
Pop3	AUSTRIA/Kramsach	1996/1997	15	5	1	-
Pop4	SWITZERLAND/Pontresina	1996/1997	6	5	-	-
Pop5	AUSTRIA/Zams	1996/1997	13	5	-	-
Pop6	AUSTRIA/Tamsweg	1996/1997	15	5	-	-
Pop7	AUSTRIA/Totzenbach	1996/1997	15	5	1	-
Pop8	GERMANY/Freiburg	1996/1997	13	5	1	-
Pop9	DENMARK/Grib Skov	1996/1997	15	5	1	-
Pop10	CHINA/Yakeshi	2001	15	5	2	-
Pop11	CHINA/Inner Mongolia, Changchun	2001	15	5	2	-
Popx	HUNGARY/Sopron	2011	-	-	-	8
Σ			**152**	**55**	**8**	**8**

3.2 DNA extraction and alkaline lysis extraction for detection of NUMTs

Total insect DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, USA). All buffers and solutions were provided with the kit. DNA extraction was performed following the protocol as recommended by the producer: each specimen was put into a 1,5 ml Eppendorf tube, overlayed with 180 µl lysis solution T (B-6678) and homogenized with a pestle. 20 µl Sigma Proteinase K were added to the mixture, incubated at 55°C for at least 2,5h and shaken at 450 rpm. After incubation RNAse was added and the mixture rested for 2 min at room temperature. 200 µl lysis solution C (B-8803) were added and the mixture incubated for another 10 min at 70°C. The DNA in the tube was precipitated by adding 200 µl ethanol and transferring the mixture onto a silica-membrane binding column. The columns were washed twice with 500 µl washing solution and DNA was eluted in 100 µl elution solution. Eluted DNA was stored at 4°C.

In order to test for the presence of mitochondrial fragments in the nuclear genome (NUMTs), an isolation of mtDNA was performed using the Tamura and Aotsuka (1988) alkaline lysis protocol, modified by Sunnucks and Hales (1996). Therefore, eight fresh *I. cembrae* beetles from Hungary were intersected alive, one body half was

subjected to this extraction method while the other half was extracted using the GenElute^{TM} Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, USA). To make sure that there was no nuclear contamination, PCR was performed with both mitochondrial *COI* and nuclear ITS2 primers. The *COI* fragments were sequenced for confirming previously derived sequences.

3.3 PCR of *Wolbachia*, mitochondrial *COI* and nuclear ITS2 markers

Four individuals from both Europe and Asia were examined for a potential presence of *Wolbachia* by performing Standard PCR amplifications of the endosymbionts *Wolbachia surface protein* primers (*wsp*) 81F and 691R described by Braig *et al*. (1998). The reactions were set up in10 µl volumes containing 1x NH_4 buffer (Fermentas), 4 mM $MgCl_2$, 200 µM dNTPs, 0,4 µM of each primer, 0,5 U *Taq* polymerase (Fermentas, Lithuania) and 1 µl of the template DNA. PCR was started for 2 min at 95°C and followed by 32 cycles at 94°C for 30 sec, 55°C for 45 sec, 72°C for 1 min and a final extension at 68°C for 15 min.

A 710 bp region of the *COI* gene was amplified for six to 15 individuals per population by using the sense primer (ItCOIF) described by Juan *et al.* (1995) and the antisense primer (UEA10) developed by Lunt *et al.* (1996). This region is situated in the second half of the *COI* gene. PCRs were run in a 25 µl total reaction containing 2 mM of $MgCl_2$, 50 mM dNTPs, 0,4 mM of each primer, 1 unit of *Taq* polymerase (Fermentas, Lithuania) and 2 µl template DNA. Thermocycling was performed in a Primus 25 thermocycler (Peqlab, Germany) in 200 µl Eppendorf tubes (Biozym, Germany). An initial denaturation at 94°C for 3 min was followed by 33 cycles (94°C for 1 min, 48°C for 1 min, 72°C for 1 min) and a final extension at 72°C for 5 min.

The full ITS2 region of five individuals per population was amplified for analyses of the nuclear genome, including the end of the 5.8S and the beginning of the 28S ribosomal gene. PCR procedure was run in a 25 µl total reaction containing 1,5 mM $MgCl_2$, 100 mM dNTPs, 0,2 mM of the primers ITS2F and ITS2R (Campbell *et al.*, 1993) and 1 unit of *Taq* polymerase (Fermentas, Lithuania) and 2 µl template DNA. Thermocycling was performed in a Primus 25 thermocycler (Peqlab, Germany) in 200

µl Eppendorf tubes (Biozym, Germany). The reaction was performed under the following conditions: denaturation step at 95°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min during 30 cycles. An initial cycle employed a 3 min denaturation at 95°C and a final cycle had an extension step of 72°C for 5 min.

3.4 Agarose gel electrophoresis, purification and DNA sequencing

Agarose gel electrophoresis was performed to make sure the PCR succeeded. For the detection of *Wolbachia* 10 µl of DNA fragments were applied on a submarine horizontal gel system using a 1x TAE running buffer. For each *COI* and ITS2 1-4 µl of PCR sample were applied, as well as an H_20_{dd} negative-PCR sample. Gels with a 1 to 2% agarose concentration and 0,5 µg/ml ethidium bromide were used. DNA was visualized on a UV transilluminator.
Purifying and sequencing of PCR products were accomplished externally by the commercial provider Eurofins MWG Operon (Ebersberg, Germany). Sequence chromatograms and sequence listings were supplied electronically.

3.5 Statistics and data analysis

COI
Nucleotide mtDNA sequences were aligned and visually optimized in a multiple alignment with 4Peaks and Seaview programme. After editing and trimming, the final sequence length used was 573 bp. Haplotypes represented by only one individual were verified by an additional PCR in order to exclude *Taq* error during amplification.

Phylogenetic analyses
Maximum-parsimony (MP) and maximum-likelihood (ML) tree reconstructions were performed with MEGA5 (Tamura *et al*., 2011). The ML trees were generated by using the model of nucleotide substitution which best fits the data under the hierarchical likelihood ratio test (hLRT) criterion, determined by MEGA5 (Tamura *et al*., 2011). A heuristic search option using 1000 random-addition sequence replicates

and explored tree space by the Tree Bisection and Reconnection (TBR) branch swapping was carried out for the MP tree reconstructions. The robustness of trees was assessed by 500 bootstrap replicates. To test constancy in rates of *COI* evolution among lineages, maximum-likelihood trees were constructed with and without a molecular clock enforced. On this reason a likelihood-ratio test (LRT; Felsenstein, 1988) was used with a homogeneous rate of evolution as null hypothesis. The LRT statistic was defined as twice the difference of log-likelihood scores from constrained and unconstrained trees, compared to a χ^2 distribution with N – 2 degrees of freedom (N = number of sequences in the tree). Uncorrected "p" genetic distances were computed using MEGA5 (Tamura *et al.*, 2011). Statistical parsimony networks were generated using TCS version 1.21 (Clement *et al.*, 2000). The five haplotypes (HTI to HTV) revealed by the former study of Stauffer *et al.* (2001) were included. Topological, geographical and frequency criteria (Crandall & Templeton, 1993) were used to solve the few cladogram ambiguities which emerged.

Population genetic parameters and analyses of population structure

ARLEQUIN (Excoffier *et al.*, 2005) was applied for calculating genetic indexes as haplotype diversity H_d (mean ± SD), nucleotide-diversity π (mean ± SD) and the mean number of pairwise differences MNPD (mean ± SD) for each population. Allelic richness r was computed for all populations after rarefaction to six by the method proposed by Petit *et al.* (1998) using the software CONTRIB (http://www.pierroton.inra.fr/genetics/labo/Software/Contrib/). Occurrence of a significant mitochondrial phylogeographic structure was assessed by testing if Gst (coefficient of genetic variation over all populations) was significantly smaller than Nst (equivalent coefficient taking into account the similarities between haplotypes), using 1000 permutations in PERMUT (see Pons & Petit, 1996) (http://www.pierroton.inra.fr/genetics/labo /Software/Permut/). Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 1992) was implemented to partition the molecular variance into different hierarchical levels by using ARLEQUIN 3.11. Samples were grouped according to the geographical range (i.e. European vs. Asian populations). The significance level of F_{ST}-statistics was determined by applying a non-parametric permutation procedure with 1000 randomizations, also generated in ARLEQUIN.

ITS2

Nucleotide sequences were aligned and visually optimized in a multiple alignment with 4Peaks and Seaview programme. After editing and trimming, the final sequence length used was 629 bp. However, the ITS2 chromatograms of most *I. cembrae* individuals displayed double peaks at two sequence positions (Figure 3.2). These double peaks suggested the superposition of two sequences reflecting heterozygosity. Two individuals from Asia showed differing sequences and were excluded from further analyses. The allele sequences from 53 individuals were reconstructed by comparing chromatograms for the forward and reverse primers following Flot *et al.* (2006). Neighbour-joining (NJ) and maximum-parsimony (MP) tree reconstructions as well as an allele network were calculated following the software and method previously defined.

Phylogenetic analyses, population genetic parameters and population structure

Uncorrected "p" genetic distances were computed using MEGA5 (Tamura *et al.*, 2011). Haplotype diversity H_d and nucleotide diversity were calculated with ARLEQUIN (Excoffier *et al.*, 2005). Allelic richness r was computed for all populations after rarefaction to four by the method proposed by Petit *et al.* (1998), using the software CONTRIB.

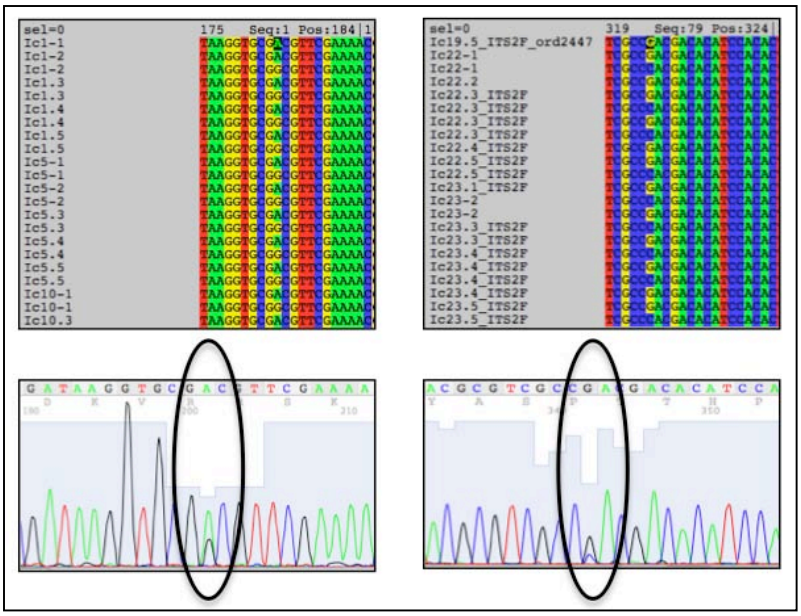

Figure 3.2: Ambigious alignments (above) and chromatograms (below) for *Ips cembrae* ITS2 at position 184 (left) and position 324 (right).

4. RESULTS AND DISCUSSION

4.1 Possible pitfalls in mtDNA: The presence of *Wolbachia* and NUMTs in *Ips cembrae*

Eight *Ips cembrae* individuals from both Asian and European populations were investigated using a *wsp* specific primer. No visible PCR product was revealed and thus *Wolbachia* was not detected by the conventional PCR method (Figure 4.1).

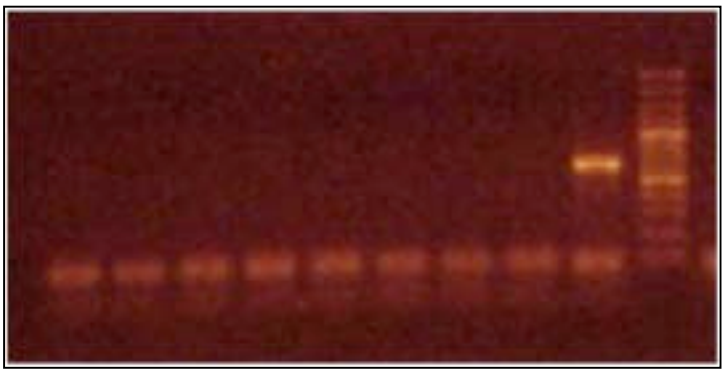

Figure 4.1: Results of agarose gel electrophoresis for detection of *Wolbachia* infection in eight *I. cembrae* individuals. Electrophoresis showed negative bands for PCR expressed *wsp* marker (from left to right). The positive band (second from right) corresponded to a *Wolbachia* infected *Rhagoletis cerasi* product which was applied for positive control. On the very right side a 1000 bp ladder was added.

In some insect species *Wolbachia* strains are only present at low frequencies (Braig *et al.*, 1998; Werren *et al.*, 2008). Low titre techniques, as for instance nested PCR or Southern hybridization technique (Arthofer *et al.*, 2009a, b), allow more sensitive investigations on the presence of *Wolbachia*. As these techniques were not applied in this study, it can not be excluded that *Wolbachia* strains are present in *I. cembrae* populations. Nevertheless it is unlikely that low titre strains or low frequency occurrence have a significant impact onto the mitochondrial genome variation (Arthofer *et al.*, 2009a, b). Moreover, given that only a small fraction of eight specimens was screened, it might be that *Wolbachia* was purely overlooked.

Thus, for future studies the above mentioned techniques could be applied by screening more individuals from European and Asian populations. The standard PCR

used in this study should be completed by long and nested PCR of other *Wolbachia* specific genes like *ftsZ* (Jeyaprakash & Hoy, 2000), 16S-DNA (Gómez-Valero *et al.*, 2004) or the five housekeeping genes developed by Baldo *et al.* (2006). Therefore, cloning and sequencing of PCR products are recommended to identify the endosymbionts strains and possible superinfections. The use of hybridization probes, specific for *Wolbachia* genes mentioned above (Gómez-Valero *et al.*, 2004), or by *in situ* immunostaining procedures (McGraw *et al.*, 2002), could also reveal new insights into the endosymbionts' presence and thus influence on *I. cembrae* populations.

As unidirectional reproductive incompatibility was for instance described in the bark beetle *Pityogenes chalcographus* (Führer, 1977), it might be interesting to investigate this potential influence of *Wolbachia* in larch bark beetles as well. However, breeding experiments with *Wolbachia* positive and negative insects are necessary to determine, whether effects like unidirectional incompatibility were exclusively caused by the endosymbiont (Werren, 1997; Arthofer, 2005). Hence, an infected laboratory stock would facilitate the development of a reliable and robust detection system for subsequent broad screening of wildlife populations, especially with respect to possible differences between infected European and Asian populations. Investigations on the geographic distribution of *Wolbachia* infections and mitochondrial haplotypes (HT) of *I. cembrae* may provide further evidence for an endosymbionts' influence on the hosts mitogenome (Turelli *et al.*, 1992; Jiggins, 2003). In order to complete the understanding of *Wolbachia* effects on bark beetles and insects in general, artifical transfers of the endosymbiont into new hosts should be performed (McGraw *et al.*, 2002; Riegler *et al.*, 2004).

Concerning NUMTs, the eight individuals extracted with both the conventional and alkaline lysis method gave clear chromatograms with neither ambiguities nor double-peaks. On the basis of these sequence chromatograms the presence of nuclear pseudogenes (i.e. the presence of mitochondrial fragments in nuclear DNA) could be excluded. Each individual had only one haplotype and thus homoplasmy was affirmed. (Figure 4.2).

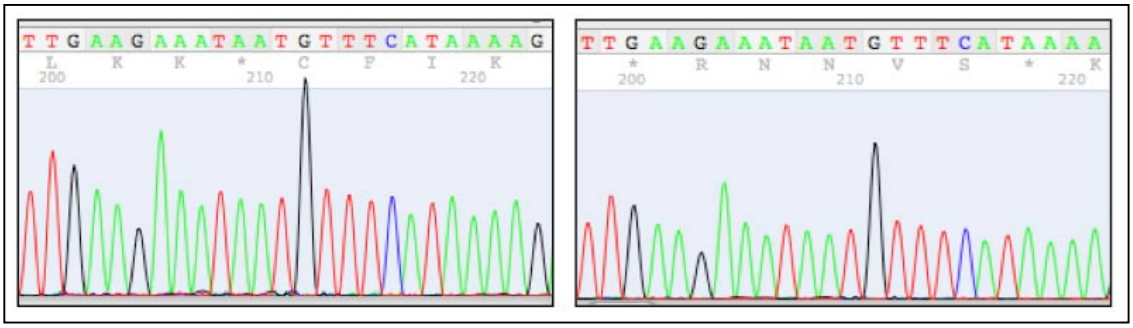

Figure 4.2: Left chromatogram derived by alkaline lysis extraction, right chromatogram derived by the conventional extraction method. 16 chromatograms obtained from eight individuals showed neither double peaks nor ambiguities.

It is generally accepted that the most common tests to safeguard against NUMTs contamination are the search for double peaks, non-synonymous mutations, indels, frameshifts and additional stop codons (Song *et al.*, 2008). With focusing on conspicuous double peaks, the results did not indicate a potential NUMTs amplification. However, as the specimens were exclusively taken from the Hungarian population, it is recommended to perform further analyses on NUMTs in European as well as Asian populations.

Recently, studies have shown that particularly the presence of cryptic NUMTs could be responsible for the putative haplotype richness of some other scolytid species, e.g. about 150 haplotypes in more than 1000 *Pityogenes chalcographus* individuals (pers. comm. Coralie Bertheau, 2011) or 131 haplotypes in 218 samples of *Dendroctonus valens* (Cognato *et al.*, 2005). The current tools for NUMTs detection and elimination are defined and widely accepted, but the evidence of cryptic NUMTs, with minimal differences to the authentic sequences, calls for attention. In a mixed template, which contains closely related sequences, the outcome of end-point PCR is a highly stochastic process and may range from complete suppression of one template to an almost 1:1 ratio in the final amplicon (Suzuki *et al.*, 1996, Schnell & Mendoza, 1997), but only the latter case will generate the suspicious double peaks which pave the way for NUMTs identification. Furthermore, enrichment of mtDNA by the alkaline lysis extraction method requires the availability of fresh tissue and is, even then, not always

reliable (Sword *et al.*, 2007, Koutroumpa *et al.*, 2009). Developments in next generation sequencing (Meyer *et al.*, 2008, Tautz *et al.*, 2010) may relax the process of NUMTs detection in the future. Until these new methods become broadly applicable, cautionary approaches like cloning and sequencing of a subset of samples should be applied, whenever an unusual richness of haplotypes is identified in a species. Alternatively, RNA extraction and RT-PCR or quantitative PCR techniques, making use of the unequal copy numbers of mtDNA and NUMTs templates, might be implemented (Bertheau *et al.*, 2011).

4.2 Phylogeography of European and Asian larch bark beetle populations

COI - Sequence alignment, phylogenetic reconstruction, genetic diversity parameters

The alignment of 573 bp sequences derived from 152 individuals from 11 populations (122 from 9 European populations and 30 from 2 Asian populations) revealed 22 haplotypes with 33 polymorphic sites (30 transitions and 3 transversions). No insertions or deletions were attested. Mitochondrial analyses yielded much higher haplotypic richness than the former study by Stauffer *et al.* (2001). As influences of both *Wolbachia* as well as NUMTs were not detected, these factors seem not to have altered the mitochondrial dataset. In fact, two European haplotypes were identical to Stauffer *et al.* (2001), namely HTI and HTII. Moreover, HT3 to HT22 were newly found haplotypes. HTI, HTII and HT3 were the most commonly detected haplotyes shared by 91, 14 and 17 individuals, respectively. In addition, six haplotypes, i.e. HT4, HT5, HT6, HT7, HT8 and HT12, were detected in two to five individuals. 13 haplotypes were found only once (Figure 4.3) and were affirmed by a second PCR. The European HTIII derived Stauffer *et al*. (2001) was not detected, even though the authours suggested a higher sample size in order to reveal this haplotype in Central Europe. The Asian HTIV and HTV, found by the former study, were not detected either.

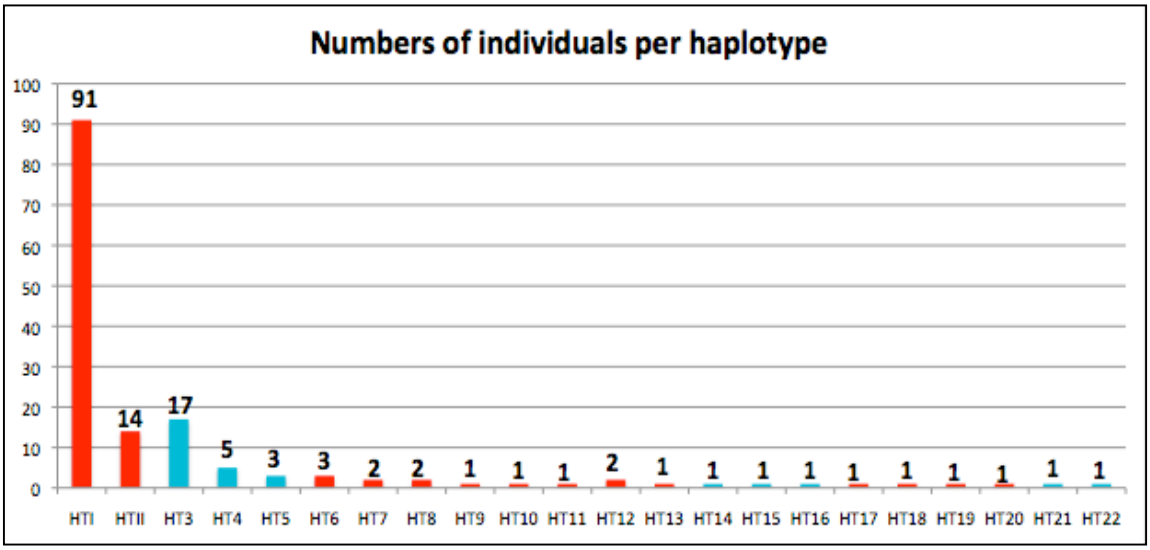

Figure 4.3: Numbers of individuals per haplotype. Haplotypes in red were only found in European populations, haplotypes in blue were exclusively found in Asian populations. HTI and HTII were also found by the former study of Stauffer *et al.* (2001).

The 22 haplotypes were joined in a single haplotype network with 95% probability (Figure 4.4). The maximum numbers of steps connecting parsimoniously two haplotypes were indicated. European and Asian haplotypes were clearly separated and did not share any haplotype. Ten European haplotypes differed in just one nucleotide position from the most frequent HTI. HT20 differed in two and HT12 in three nucleotide positions from HTI. Two out of seven haplotypes differed in just one nucleotide position from the most frequent Asian HT3. Five haplotypes differed in two or more nucleotide positions. HTIV and HTV, which are the former Asian haplotypes by Stauffer *et al.* (2001), were located closer to current European haplotypes than to current Asian haplotypes, as they differed in one and four nucleotide positions from the second most frequent European HTII.

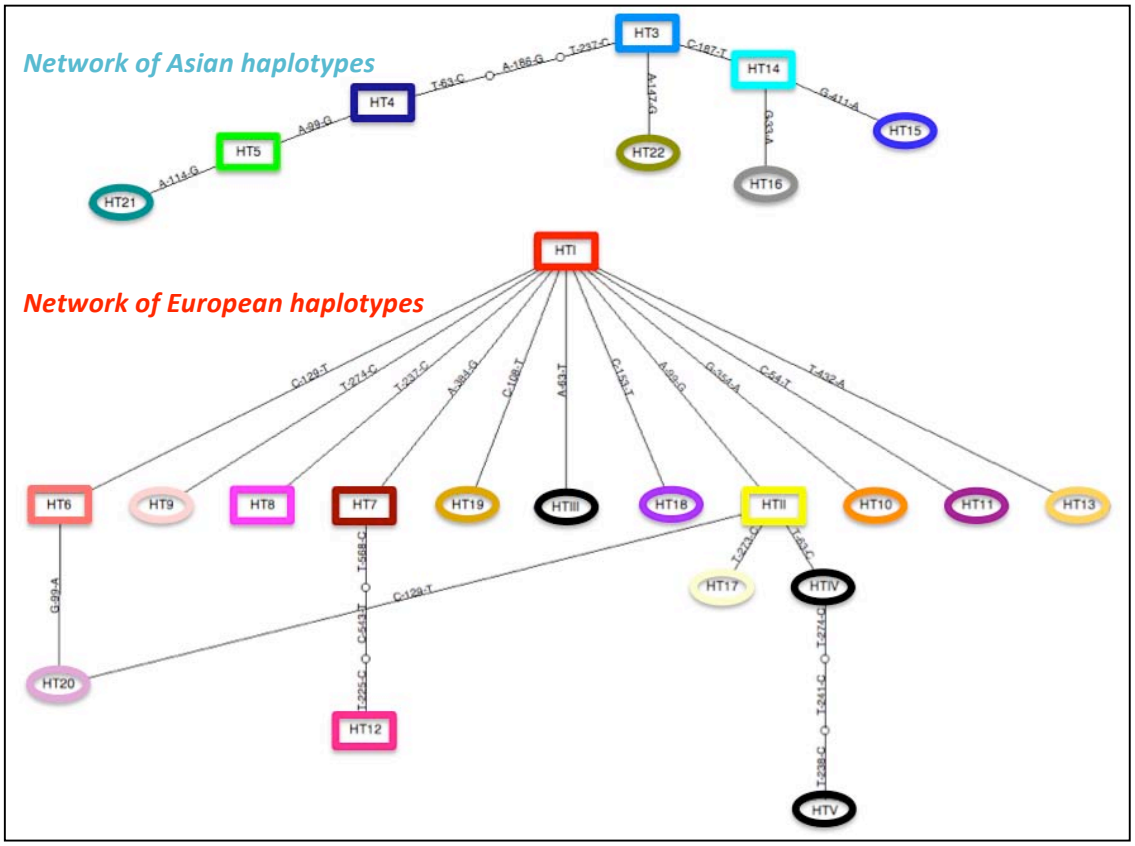

Figure 4.4: Haplotype network containing the 22 haplotypes found in this study. Each line corresponds to a mutational step and each empty circle to a missing intermediate. HTs in oval shape are found only once, HTs in rectangle shape were shared by more than one individual. HTIV to HTV described by Stauffer *et al.* (2001) were added in black oval shapes. The upper network corresponds to clade B and thus to Asian haplotypes, calculated by the MP tree shown in figure 4.5; the lower network corresponds to clade A, which grouped the European haplotypes.

The hierarchical likelihood-ratio tests using MEGA revealed, that the most appropriate model of sequence evolution was T92+G+I model, including invariable sites (I=0.87) and rate variation among sites (G = 1.67), with unequal base frequencies (freqA = 0.349; freqC = 0.151; freqT = 0.349; freqG = 0.151). The likelihood-ratio test supported a molecular clock model for *I. cembrae* (χ^2 = 35.02, d.f. =20, P < 0.05). Phylogenetic reconstructions, both MP and ML, yielded in congruent trees with two clades labeled A and B (Figure 4.5). Results illustrated that the 14 European haplotypes and the eight Asian haplotypes were monophyletic. Populations were placed in two distinct clades, each supported by high bootstrap values. Clade A exclusively contained European populations, while clade B clustered populations

from Asia. P-distance calculated between the two clades ranged from 0,22% (e.g. between HT1 and HT3) to 4,5% (e.g. between HT11 and HT22), with an average of 2,2% (Table 4.1).

Table 4.1: Computed distance matrix of *COI* based on p-distance

Group	Average	Max	Min
All	0,022 (2,2%)	0,045 (4,5%)	0,002 (0,2%)
Europe	0,004 (0,4%)	0,010 (1%)	0,002 (0,2%)
Asia	0,006 (0,6%)	0,012 (1,2%)	0,002 (0,2%)

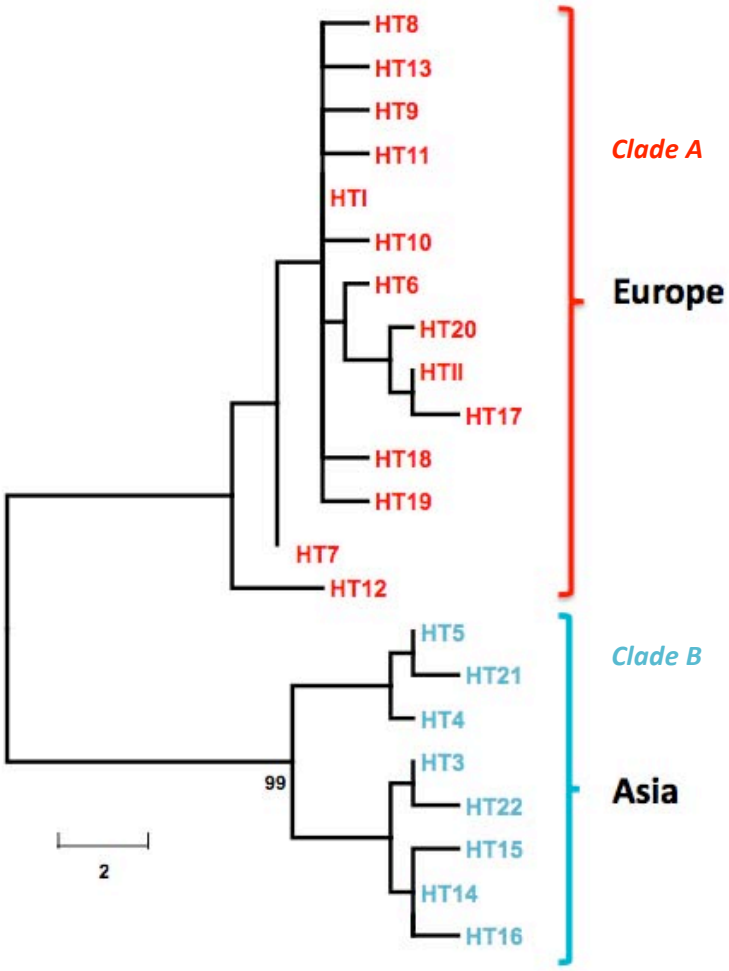

Figure 4.5: Maximum Parsimony tree of 22 haplotypes found in *Ips cembrae* populations. Bootstrap values over 95% are given above nodes. Clade A exclusively clustered haplotypes from Europe, whereas clade B contained haplotypes from Asia. Both Clades were monophyletic.

The separation time between the two lineages was estimated by using the general molecular clock estimate for Coleopteran mitochondrial genes, calibrated from other Cerambycidae belonging to the genus Tetraopes (1,5% per million years) (Farrell, 2001). Considering the genetic distances observed between the two lineages, analysis implied an average divergence time of 1,47 to 3 million YBP. Despite the lack of precision of such estimates, it can be concluded that the separation between the European and Asian populations was of late Pliocene origin, the beginning of the Pleistocene period.

The geographical distribution of a total of 22 haplotypes is displayed in figure 4.6. HTI was the most frequent haplotype found in Europe, occurring in each European population, followed by the second most frequent European haplotype, namely HTII. Especially the northern population in Denmark, where the insect was introduced by timber trade (Redfern *et al.*, 1987), was more weakly differentiated than most populations in Central Europe and Slovenia, where several single haplotypes were found. The population in Denmark revealed only HTI and HTII, which were in fact the most frequently found haplotypes in Europe. Contrary to Stauffer *et al.* (2001), these findings could concur with Hewitt's hypothesis (1996), which suggests that less haplotypes are found in areas of recent introduction. The Swiss population (Pop4, Pontresina), the population in northeastern Austria (Pop7, Totzenbach) and the Slovenian population (Pop2, Martuljek) showed the highest number of four haplotypes per population. Moreover, the highest number of three single haplotypes per population was detected in the Swiss population (Pop4, Pontresina). Compared to relatively low structured populations in Europe, the haplotypic richness in Asian populations indicated higher varibility among Asian larch bark beetles. The two populations investigated shared HT3 and HT4. Four haplotypes were found in the northeastern Chinese population (Pop10, Yakeshi) and six haplotypes were found in the Inner Mongolian population (Pop11, Changchun), whereas the first population revealed two single haplotypes and the latter three single haplotypes, respectively.

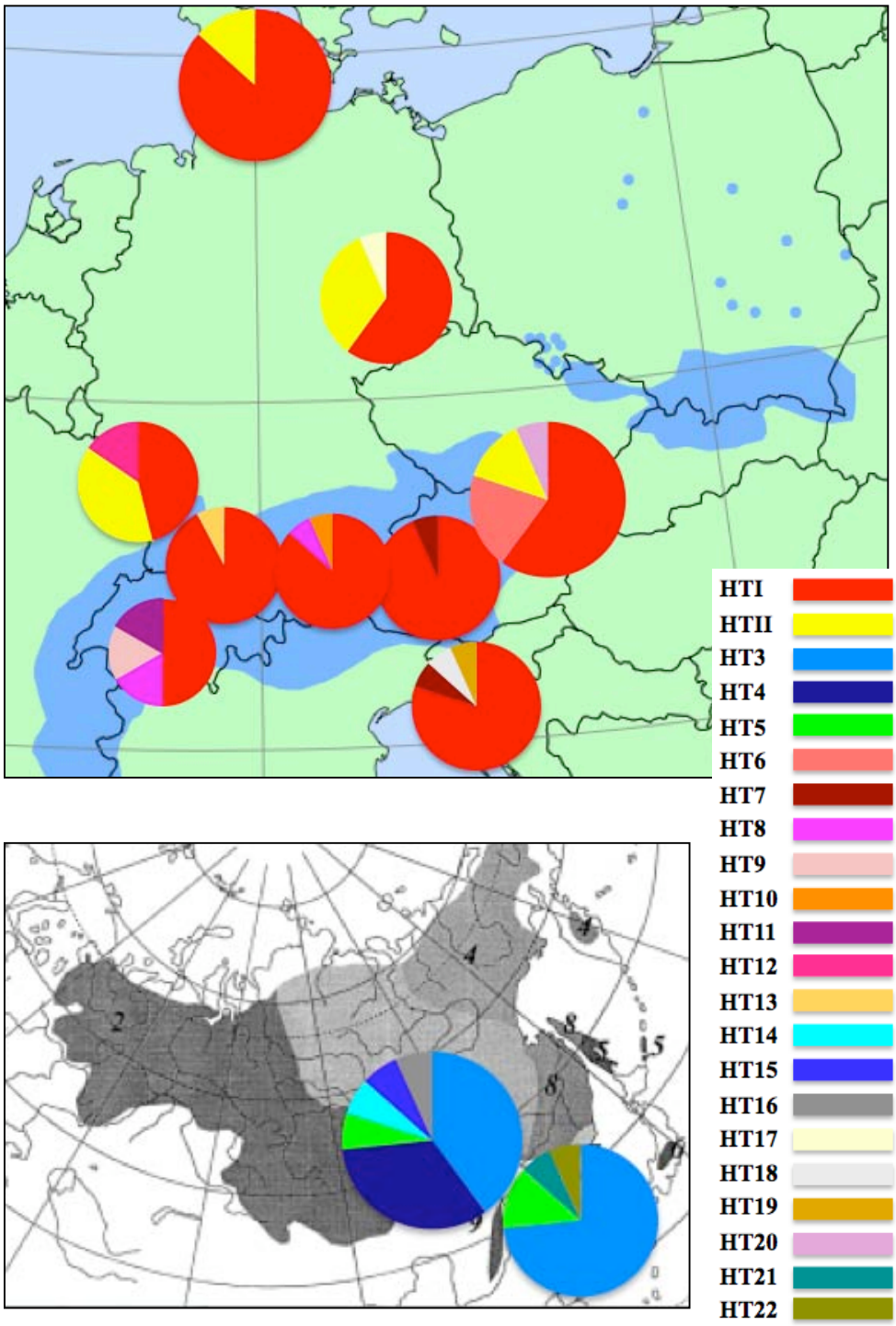

Figure 4.6: Haplotypes distribution of European and Asian *I. cembrae* populations.

Occurrence of a significant mitochondrial phylogeographic structure was assessed by testing if Gst (coefficient of genetic variation over all populations) was significantly smaller than Nst (equivalent coefficient taking into account the similarities between haplotypes). Total gene diversity Ht was 0,644, whereas the average within-population diversity Hs was 0,468. The indices of population structure Gst and Nst were 0,288 and 0,898, respectively. Gst was significantly smaller than Nst, indicating a significant structuration and genetic difference between European and Asian populations. The within-population diversity indices, i.e. gene diversity H_d, allelic richness r, nucleotide diversity π and mean number pairwise differences MNPD, are given in table 4.2.

Table 4.2: Within-population diversity indices for European and Asian *I. cembrae* populations. Populations are coded Pop1-Pop11; European populations are shown in red, Asian populations in blue. Numbers in brackets after haplotype names stand for numbers of individuals sharing this haplotype.

Population	N	#HT	Haplotypes	Hd ± SD	MNPD ± SD	π ± SD	r (6)
Pop1	15	3	HTI(9); HTII(5); HT17(1)	0,562 +/- 0,095	0,648 +/- 0,531	0,001 +/ 0,001	1, 36 (6)
Pop2	15	4	HTI(12); HT7(1); HT18(1); HT19(1)	0,371 +/- 0,153	0,4 +/- 0,392	0,001 +/- 0,001	1,2 (6)
Pop3	15	3	HTI(13); HT10(1); HT8(1)	0,257 +/- 0,142	0,267 +/- 0,309	0,0005 +/- 0,0006	0,8 (6)
Pop4	6	4	HTI(3); HT11(1); HT8(1); HT9(1)	0,8 +/- 0,172	1 +/- 0,775	0,002 +/- 0,002	3 (6)
Pop5	13	2	HTI(12); HT13(1)	0,154 +/- 0,126	0,154 +/- 0,229	0,0003 +/- 0,0005	0,46 (6)
Pop6	15	2	HTI(14); HT7(1)	0,133 +/- 0,112	0,133 +/- 0,21	0,0002 +/- 0,0004	0,4 (6)
Pop7	15	4	HTI(9); HT6(3); HTII(2); HT20(1)	0,619 +/- 0,12	0,762 +/- 0,591	0,001 +/- 0,001	1,87 (6)
Pop8	13	3	HTI(6); HTII(5); HT12(2)	0,667 +/- 0,078	1,641 +/- 1,033	0,003 +/- 0,002	1,71 (6)
Pop9	15	2	HTI(13); HTII(2)	0,248 +/- 0,131	0,248 +/- 0,296	0,0004 +/- 0,0006	0,66 (6)
Pop10	15	4	HT3(11); HT5(2); HT21(1); HT22(1)	0,467 +/- 0,148	1,638 +/- 1,022	0,003 +/- 0,002	1,46 (6)
Pop11	15	6	HT3(6); HT4(5); HT5(1); HT14(1); HT15(1); HT16(1)	0,762 +/- 0,081	2,286 +/- 1,327	0,004 +/- 0,003	2,54 (6)
			HTI(91), HTII(14), HT6(3), HT7(2), HT8(2), HT9(1), HT10(1)				
European Pop	122	14	HT11(1), HT12(2), HT13(1), HT17(1), HT18(1), HT19(1), HT20(1)	0,432 +/- 0,054	0,602 +/- 0,483	0,001 +/- 0,001	
Asian Pop	30	8	HT3(17), HT4(5), HT5(3), HT14(1), HT15(1), HT16(1), HT21(1), HT22(1)	0,66 +/- 0,087	1,995 +/- 1,158	0,003 +/- 0,002	
Σ	152	22					

N: Number of individuals analysed
#HT: number of HT per population
H: Gene diversity and its standard deviation
r: Allelic richness after rarefaction to 6
π: Nucleotide diversity and its standard deviation
MNPD: Mean number pairwise differences and its standard deviation

Although the genetic subdivision in European populations was overall low, the value of gene diversity among populations strongly varied. The Swiss population (Pop4, Pontresina) showed the highest within-population diversity in European populations, i.e. 0,8. HT8, HT9 and HT11 were exclusively found in this population and thus

contributed to the high gene diversity. According to Wood & Bright (1992) as well as to Pfeffer (1995), *I. cembrae* var. *engadiensis*, mainly infesting *Picea abies*, is assumed to be present in Switzerland (see chapter 1.2). It is worth noting that Stauffer *et al.* (2001) intended to consider these references by focusing on haplotypes revealed from Swiss specimens. Back then the authors were not able to detect any unique haplotype and thus no host speciation could be deduced from the former data in 2001. Interestingly, the current results indicated that the Swiss population is the most differentiated population in Europe by revealing three single haplotypes. However, regarding the small sample size of Swiss specimens as well as the lack of information about host trees, it can not be determined whether the high output of unique haplotypes emphasizes the presence of *I. cembrae* var. *engadiensis*. Despite that, investigations intending to deliver further insights into host speciation should incorporate the data presented in the current study.

The most weakly differentiated populations were the Central Austrian population (Pop6, Tamsweg) and the Danish population (Pop5, Grib Skov) with 0,133 and 0,154, respectively. The Asian Population in Inner Mongolia (Pop11, Changchun) showed higher gene diversity than the northeastern Chinese population (Pop10, Yakeshi). The average gene diversity within all Asian populations was 0,66 and thus much higher than the gene diversity within all European populations, which was 0,432.

In respect of the results, further research should particularly focus on the intraspecific differentation of Asian larch bark beetles. The sequences divergence as well as the number of haplotypes derived from Asian populations were remarkably high, regarding the small sample size of 30 individuals. The average sequence divergence of 0,6% between Asian populations was higher than the sequence divergence found between European populations, which was 0,4% (Table 4.1). Considering the four times higher sample size of European specimens (122 individuals) it can be presumed, that Asian *Ips subelongatus* features a much higher genetic variability than revealed so far. Quite recently a study explored the insects behavioural responses to potential aggregation pheromone components in populations located in two provinces of northeastern China (Song *et al.*, 2011). Results indicated a strong geographical variation and disparity in aggregation pheromone response of *I. subelongatus*,

supporting the current findings of high genetic subdivision in Asian populations. Results of AMOVA analyses are summarized in table 4.3.

Table 4.3: Results of AMOVA analyses: * significant, ** highly significant

	Source of variation	Variance components	Percentage of variation
Grouping by region	Between groups	11,168 Va	96,20% *
	Between pops within groups	0,031 Vb	0,26% *
	Within populations	0,411 Vc	3,54% **

When populations were geographically grouped, the main part of variability observed was due to the genetic difference between European and Asian groups (i.e. 96%). AMOVA Analysis showed that there was an overall low genetic variance among populations of each group, as variation between European as well as between Asian populations was 0,26%. Moreover, even the variation within populations (i.e. the genetic difference found between the individuals of a specific population) was 3,54% and thus very low.

However, as indicated by the 96% variation between groups, there is an evidence for structuration by distance between European and Asian larch bark beetle populations. The phylogenetic reconstruction as well as genetic diversity parameters suggested that there is no gene flow between these two geographic entities.

ITS2 - Sequence alignment, phylogenetic reconstruction and diversity parameters

The alignment of 629 bp of 55 individuals from 11 populations (45 individuals from 9 European populations and 10 individuals from 2 Asian populations) revealed four different alleles coded allele-1 to allele-4. Alleles were determined due to two polymorphic sites (one transition at position 208, one transversion at position 249) and to two ambigious positions (184 and 324) (Table 4.4).

Table 4.4: Alleles found in *I. cembrae* ITS2 sequences.

Pos.	184	208	249	324
Allele-1	A	T	A	G
Allele-2	G	T	A	G
Allele-3	A	C	C	G
Allele-4	A	C	C	C

Analyses of two Asian individuals revealed different results, as the chromatograms emerged ambigious peaks at position 147 (C/T). These findings could be taken as a hint for possibly more allelles occurring in Asia, even though various explanations could be mentioned, for example multicopy in ITS2. For obtaining clear results about potential alleles, cloning might be suggested. However, allele-1 and allele-2 exclusively occurred in European populations, whereby allele-3 and allele-4 were only found in Asian samples (Figure 4.7) and thus conveyed complementary information to mitochondrial outcomes.

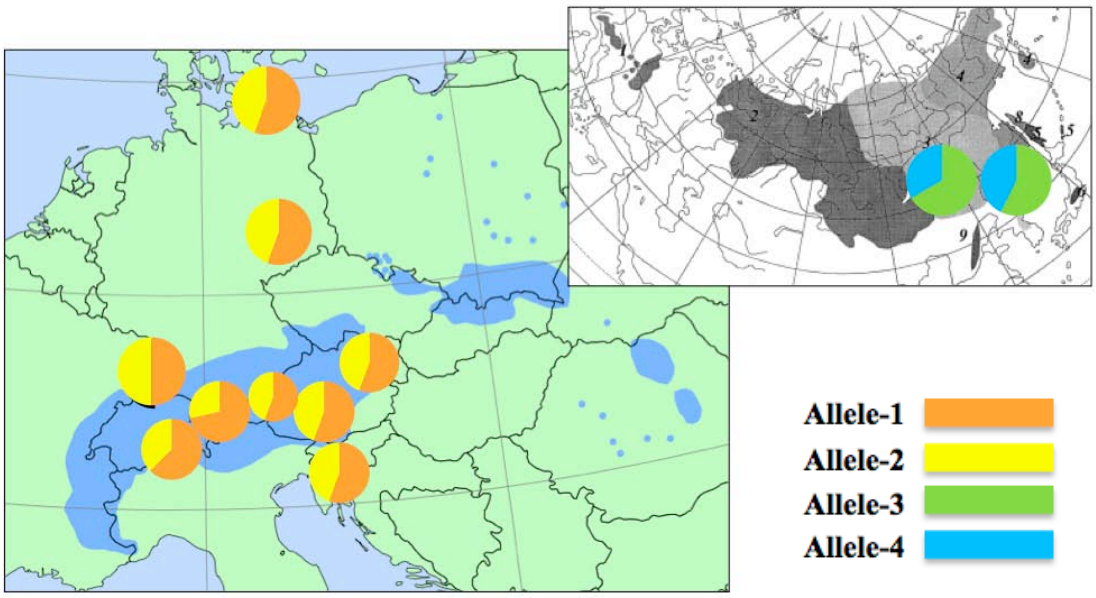

Figure 4.7: Distribution of ITS2 alleles in European (left) and Asian (right) populations.

Both NJ (Figure 4.8) and MP reconstructions yielded congruent trees with two lineages, which were supported by high bootstrap values. One lineage grouped the two European alleles, while the other lineage included the two Asian ones.

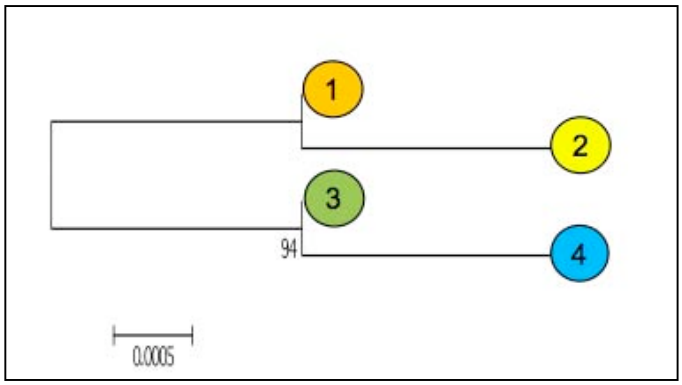

Figure 4.8: Neighbour-Joining tree of four alleles found in *I. cembrae*, based on the Kimura 2 Paramters model.

P-distance calculated between the two clades ranged from 0,2% to 0,6%, with an average of 0,38%. In each group the p-distance was 0,2%, wheareas divergence between the two groups ranged from 0,3% to 0,6%. The proportion of different alleles in the samples yielded a total gene diversity and nucleotide diversity of 0,63 and 0,002, respectively. Within-population gene diversity and allelic richness r are given in table 4.5.

Table 4.5: Within-population diversity indices of European and Asian populations derived by ITS2 analyses.

Population	Na	Gene Diversity (Hd)	Allelic Richness (r)
Pop1	2 (1, 2)	0,56 +/- 0,09	2
Pop2	2 (1, 2)	0,56 +/- 0,07	2
Pop3	2 (1, 2)	0,56 +/- 0,05	2
Pop4	2 (1, 2)	0,54 +/- 0,12	2
Pop5	2 (1, 2)	0,48 +/- 0,17	1,978
Pop6	2 (1, 2)	0,56 +/- 0,05	2
Pop7	2 (1, 2)	0,56 +/- 0,05	2
Pop8	2 (1, 2)	0,56 +/- 0,05	2
Pop9	2 (1, 2)	0,56 +/- 0,05	2
Pop10	2 (3, 4)	0,71 +/- 0,12	2
Pop11	2 (3, 4)	0,71 +/- 0,10	2

Na: Number of alleles
r: Allelic richness after rarefaction to 4

Moreover, the distribution and abundance of alleles confirmed the outcomes of mtDNA analyses. Beetles belonging to the European clade A consistently shared allele-1 and allele-2, while beetles belonging to the Asian clade B shared allele-3 and allele-4. Results of mitochondrial as well as nuclear DNA examinations indicated that there is no gene flow between European and Asian *Ips cembrae* populations.

As recently argued by Schultz & Wolf (2009), the ribosomal ITS2 gene is one of the most appropriate markers in phylogenetics. In this thesis, the nuclear marker was applied onto *I. cembrae* populations for the first time. Arthropod studies revealed different patterns of variability with ITS. For example no variation in the consensus ITS2 sequences of individuals sampled worldwide was reported in the spider mite *Tetranychus urticae* (Navajas *et al.*, 1998). In contrast, analyses of the mitochondrial *COI* marker revealed a genetic diversity of 5%. Beyond that, investigation on two other mite species with greater specificity, stricter ecological requirements and more restricted colonization potential, showed substantial and concordant geographical differentiation for both ITS2 and *COI* (Navajas *et al.*, 1998). Low intraspecific variation at the ITS2 locus was also detected in sister species of *Anopheline* mosquitoes (Prakash *et al*., 2006) and *Drosophila* (Schlötterer *et al.*, 1994). Furthermore, Zhang *et al.* (2004) investigated the taxonomic status of two black widow species. Results indicated that minimal differences present in the ITS2 sequences were taxonomically insignificant. However, a substantial intra-individual heterogeneity of ITS sequences has been reported in two species of mosquitoes (Wesson *et al.*, 1992), in blackflies (Tang *et al.*, 1996) and in the tiger beetle (Vogler & DeSalle, 1994). In addition, Kerderlhué *et al.* (2002) observed that mtDNA haplotypic groups of *Tomicus piniperda* consistently differed in the length of ITS1. In fact, the current findings of this study also confirm heterogeneity of ITS2 sequences, as analyses of European and Asian larch bark beetle populations revealed four distinct alleles.

Results did not draw a clear-cut picture of phylogeographic patterns, i.e. glacial refugia or postglacial migrations routes of Palaearctic *I. cembrae*. Data demonstrated that European HTI and HTII correlated with the most frequent haplotypes described by the former study in 2001. HTI was present in each European population whereas HTII was mainly found in populations north of the Alps. It can be assumed that HTI

survived glaciation next or close to its host in refugial areas in Poland and in the southwest Alps, wherefrom it dominantly recolonized Europe. Due to the distribution of haplotypes it might be tempting to expect that HTII survived glaciation in refugia near Poland or the Tatra region, and that beetles spread only westwards and northwards of the Alps. However, the lack of HTII in populations of the central and southern Alps could indicate a bottleneck effect, as the Alpine barrier impeded a recolonization southwards. Further investigations on populations in the Carpathian and Tatra region might reveal more information on this consideration, as in particular the scattered distribution of the host *L. decidua* and its subspecies *L. decidua* ssp. *polonica*, respectively, suggests a higher intraspecific differentation of beetles throughout these regions.

Indeed, the highly diverse genetic constitution of Asian populations stands in contrast to less differentiation in European populations. These results could rely on a general assumption about European phylogeography, namely that just a few glacial refugia, together with a high number of bottleneck events during postglacial recolonization, resulted in low differentiated gene pools of species, above all compared to Asian biota. Regarding the phylogeography of *Ips subelongatus* it was also very likely that numerous small-scale refugia existed during glacial periods in Asia, where *Larix* ssp. and the beetle survived, diverged and started to recolonize. Therefore, the post-glacial genesis of the Asian larch bark beetle and its hosts might have occurred through expansion in the size and density of numerous isolated tree population patches. However, more specimens from crucial regions as the Carpathian and Tatra mountains as well as many more populations from Russia, Mongolia and China have to be analyzed in order to reveal further insights into phylogeographic events of Palaearctic larch bark beetles.

Additionally, it is important to note that *Larix* ssp. are not the only host plants associated with *I. cembrae* and *I. subelongatus*. It could be hypothesized that the phylogeography of larch bark beetles was less dependent on the main hosts *Larix* ssp. Investigations on interactions between larch bark beetles and potential host tree species, like *Pinus* ssp., *Picea* or *Abies* ssp., could open up new evidences on migration patterns and evolutionary modifications. Crucially, the role of host tree species should also be taken into account when referring to the genetic subdivision found in Asian larch bark beetle populations. The high gene diversity and relatively

high output of single haplotypes could be favoured by numerous potential host tree species, as Asian flora features a much higher number of *Larix* taxa than the European one. In regard to forest ecosystems, just a few studies exist concerning the role of host plants in the genetic structure of associated insects (Kerdelhué *et al.,* 2002). For instance, a study on the European larch budmoth *Zeiraphera diniana* indicated that the larch and pine-forms are genetically very different (Emelianov *et al.,* 1995). This result was contrary to the genetic structure of *Dendroctonus brevicomis* (Scolytinae) in the USA, which is mainly due to geographical isolation and very weak host effects (Kelley *et al*., 1999). In contrast, two studies found evidence of host effects on the genetic structure of pine beetle *Dendroctonus ponderosae* by investigating allozyme data (Sturgeon & Mitton, 1986; Kelley *et al*., 2000). However, most studies investigating the interactions of bark beetles and host trees pointed out, that host trees could not affect the structure of beetle populations (e.g. Horn *et al.,* 2006 & 2009 with *Tomicus* species; Bertheau *et al.,* 2011 pers. comm. with *P. chalcographus*; Cognato *et al.,* 2003 with *Ips confusus*, Avtzis *et al.,* 2008 with *Polygraphus grandiclava*).

4.3 The genetic relationship between European and Asian larch bark beetles

The use of DNA to help resolving phylogenetic, population genetic and taxon identification problems is common practice (e.g. Gullan *et al*., 2003; Moritz & Cicero, 2004; Miller *et al*., 2005). Several studies interested in taxonomy of insects have advocated the mitochondrial locus *COI* as the ideal gene for species identification (c.f. Cognato & Sun, 2007; Savolainen *et al.,* 2005). Therefore, it has been proposed that species boundaries can be identified by a predetermined, generally applied number of nucleotide differences between *COI* haplotypes (c.f. Coganto & Sun, 2007). In this context Hebert *et al.* (2004) suggested that ten times the mean number of nucleotide differences observed within related species can be taken to classify a taxon as a new species. Analyses of European and Asian larch bark beetles revealed an average intraspecific *COI* nucleotide difference of 0,4% and 0,6%, respectively. If the suggested ten times intraspecific *COI* divergence of Hebert *et al.* (2004) is implemented as a basis to identify species boundaries between those two geographic entities, the clades exhibiting greater than 4% (and 6%) divergence compared with sister clades would be recognized as species. In fact, the sequence divergence between

the European and Asian clade ranged up to 4,5% and would approve the suggestion of Hebert *et al.* (2004). Moreover, Brower (1994) suggested a sequence divergence higher than 2,5% in order to show speciation between two arthropod entities. With reference to this, the sequence divergence of 4,5% indicated that Asian and European larch bark beetle populations have been separated for a long time, and that they now represent distinct gene pools. However, any standard mean difference of *COI* genes ignores the range of intraspecific nucleotide difference values, which varies between 0 and 10% for *Ips* (Cognato & Sun, 2007). Hence, it is ill-advised to make taxonomic decisions solely based on mean nucleotide differences. Molecular classifiers may additionally yield a certain probability, that two organisms belong to distinct species (Müller *et al.*, 2007), but identifying new species should not only rely on molecular investigations. Moreover, the use of standards as standard mean differences would ignore years of taxonomic and biological research (see Wood & Bright, 1992 for references). Even if molecular techniques, like barcoding, provide convenient methods for delimiting taxonomic boundaries (Moritz & Cicero, 2004), studies on morphological traits should not be omitted.

For further investigation on the species status of European and Asian larch bark beetles it might be helpful to detect geographic boundaries by surveying a transect running east-west throughout Eurasia. Examinations of hybrid zones, where beetles but also *Larix* ssp. from Europe and Asia intermix, could deliver novel information on the phylogeography as well as phylogenetics of *I. cembrae*. Future studies should also focus on biological approaches (c.f. Mayr, 1982) and it would be interesting to see whether matings between European and Asian beetles produce viable and perhaps even fertile offspring. Therefore, crossing experiments might be appropriate for examining prae- or postzygotic barriers and thus for explicitly testing the species status between *I. cembrae* and *I. subelongatus*.

However, the current study delivers ample evidence to suggest *I. subelongatus* as a valid species designation for Asian larch bark beetles. Both the high mitochondrial *COI* sequence divergence between European and Asian populations as well as the clear distribution of nuclear alleles derived by analyses of ITS2 markers support the recognition of *I. cembrae* and *I. subelongatus* as two taxonomic units. But as mentioned above, studies on beetles taxonomy have rarely been explicit about what

species represent or how to delimit them. Regarding this lack of explicitness, the current outcomes may encourage future research on the *I. cembrae* complex. Besides, further investigation on discrete units of European and Asian larch bark beetles may refer to a study recently published by Müller *et al.* (2007). The authors pointed out that the presence of a compensatory base change (CBC) in the helix II or helix III ITS2 secondary structure between two organisms correlated with sexual incompatibility. The results indicated that a CBC in a pair of sequences was positively correlated with distinct, traditional species at a confidence level of 93%. Therefore, a CBC in an ITS2 sequence–structure alignment pair constitutes sufficient condition for distinguishing and delimiting even closely related species. Given that this study on Palaearctic *I. cembrae* showed an explicit heterogeneity in ITS2 sequences, the method described by Müller *et al.* (2007) might be very appropriate for reinvestigating the taxonomic status of *I. cembrae* and *I. subelongatus*.

Results on the genetic structure or geographical disparity of *I. cembrae* and *I. subelongatus* may facilitate the decision making in sustainable forest management. Prognoses concerning climate change include for instance an increase in the frequency of drought periods or wind throws (Englisch *et al.*, 2011), and thus may challenge forest protection services for observing and monitoring secondary pests like bark beetles.

As compared with European *I. cembrae*, the Asian larch bark beetles have greater economic impact in silviculture and are reported to be more damaging to the Asian local *Larix* species and more in need of control (EPPO, 2005). Concerning central European larch forests and other areas in Europe where *Larix* ssp. are afforested and *I. cembrae* can be found, *I. subelongatus* might constitute an additional risk (EPPO, 2005). Questions of quarantine are substantiated by a considerable increase in export of conifer wood from Russia and Asia to Western Europe. Moreover, *I. subelongatus* also poses a risk to other continents where Asian lumber trade is forced and *Larix* plantations are exploited, as for example in North America (EPPO, 2005). The introduction of *I. cembrae* into Asia or *I. subelongatus* into Europe as a result of international trade could have serious consequences. The threat would be caused not only by the beetle, but also its fungal associates. For instance *Ophiostoma ulmi*

(Buismann) Nannfeldt and *Ophiostoma novo-ulmi* Brasier, which are fungal associates of the elm bark beetles belonging to the genus *Scolytus*, were the causal agents of two tremendous pandemics of Dutch Elm Disease in the last century (Brasier, 1991). Pathogen/host tree interactions are often favoured in new environments as the hosts could be more susceptible (Brasier, 2000). It is highly recommended that great care should be taken to avoid the introduction of *I. cembrae* and *I. subelongatus* and their associated fungi into areas outside their natural range (Stauffer *et al.,* 2001). Therefore, information on the genetic structure of the insect, its associated pathogens and its hosts, might support biological and chemical control mechanism as well as phytosanitary measures.

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