History of the European larch (*Larix decidua* Mill.)

Doctoral Thesis

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Mit all meiner Freundschaft,
in Erinnerung der schönen Momente
mit Wolfgang

Avec toute mon amitié,
en souvenir des bons moments
passés avec Wolfgang

∞

SUMMARY

This thesis focuses on the consequences of past climate and anthropogenic changes on populations of the European larch (*Larix decidua* Mill.) by integrating palaeoecological and genetic data. Such retrospective approaches provide a useful context for evaluating possible impacts of ongoing changes. A limitation of current studies dealing with forest trees is that they often deal exclusively with postglacial recolonization. Effects of more rapid changes on forests, including those caused by recent plantations or by abrupt climatic events of the last glacial, have been largely neglected. In this study high resolution genetic data and precise vegetation records correlated with high-resolution climate records of the last interglacial/glacial cycle (130,000 years) were used to precisely document long-term and short-term events that impacted the history of European larch. For the genetic analysis, highly informative nuclear markers (microsatellites) were designed and applied on a range-wide sample of 45 modern larch populations. These data were analysed together with mitochondrial data to establish a baseline for studies focussing on recent translocations. Results revealed that larch has been planted extensively, generating admixture between native and non-native populations from multiple sources across the range. Translocation events and admixture rates were distributed unevenly across the range, with a particularly high frequency in Poland, Slovakia and the Czech Republic where larch has a more scattered distribution compared to the Alps. Some of the most valuable populations appear to be seriously endangered by translocations. The palaeoecological results showed that larch persisted close to its modern distribution throughout the last interglacial/glacial cycle but that its range was highly dynamic and in equilibrium with both long-term and short-tem climate events, in line with the pioneer character of the species. The extent of species distribution was maximal during the first early Weichselian interstadial when larch built boreal forests in the north-central European lowlands (87,000 – 109,000 years ago). Responses to short-term climate events (Dansgaard-Oeschger cycles, Heinrich Events) were extremely rapid. Seven Last Glacial Maximum (LGM) refuges were detected using fossils and genetic data. This made it possible to identify recolonization pathways and concomitant introgression and homogenisation, highlighting the power of the joint population genetic and palaeoecological perspective.

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CHAPTER 1: General introduction and acknowledgements

Scientific background

Studies of the consequences of past climate change on forests worldwide provide a necessary historical context for evaluating the possible consequences of ongoing climate change, an emerging field of research (e.g. Colombaroli *et al.* 2010; Petit *et al.* 2008; Tinner & Ammann 2005). For instance, palaeoclimatic studies have identified periods of rapid changes, with temperature shifts up to 16°C on a centennial or even decadal timescale (Wolff *et al.* 2010). Reconstructions of past forest distributions and dynamics can advance our understanding of relevant processes such as tree colonization, adaptation, and extinction in response to climatic change. A detailed understanding of these processes is necessary to accurately model future vegetation dynamics under climatic conditions for which no modern analogues exist. The advantages of such retrospective approaches are obvious in view of the difficulties to experiment with forests and climate.

In Europe, the availability of large amounts of fossil data coupled with modern reconstruction and dating techniques have enabled detailed and robust reconstructions of the last glacial and postglacial vegetation and climate based on pollen and macrofossils. Similarly, phylogeographic studies and population genetic surveys of several dominant forest trees (e.g. *Quercus*, *Fagus sylvatica*, *Pinus sylvestris*, *Abies alba*, *Picea abies*) have been performed. These studies have helped identifying glacial refugia and postglacial colonisation routes (reviewed in Hu et al. 2009). Side-by-side comparisons of genetic and fossil data have been used to reconstruct the history of these tree species since the last glacial maximum (Cheddadi *et al.* 2006; Liepelt *et al.* 2009; Magri *et al.* 2006; Petit *et al.* 2002; Tollefsrud *et al.* 2008). In comparison, the effects of more rapid changes on forests, including those caused by recent plantations or by abrupt climatic events, have been largely neglected. New, more powerful genetic markers (e.g. Guichoux *et al.* 2011a) and precise vegetation records that can be correlated to high-resolution climate records of the last glacial cycle (Fletcher *et al.* 2010) must now be used and combined to assess long-term and short-term history using different perspectives.

For such studies, suitable biological models are needed. Conifers combine several important advantages. They are typically abundant and represent keystone species for vegetation reconstruction. Thanks to their large biomass, they are susceptible to leave many fossils in sediments. In particular, these wind pollinated species produce considerable amounts of pollen that can be identified in sediments from peat bogs, ponds or lakes to reconstruct in detail their past distribution through time. Another advantage is that they have three different

genomes, each with a specific mode of inheritance (biparental for the nuclear genome, maternal for the mitochondrial genome and paternal for the chloroplast genome), which provide contrasting and complementary information on their history if the data is appropriately analysed. They are therefore very promising candidates for the application of such an approach. They should provide precise and accurate insights into past vegetation dynamics and help identify the underlying processes and lead to important improvements of existing vegetation and climate models for simulating future scenarios.

We propose as case study the European larch (*Larix decidua*). This deciduous conifer species has not been subject to recent historical studies, but it has some unique features that make it very suitable to study recent and ancient history (see chapters 2, 4 and 5).

Structure of the thesis

This thesis is an interdisciplinary study joining population genetics and palaeoecology. Prior to this work, knowledge on the range-wide genetic structure of the species was very limited. To prepare the population genetic part, I undertook a detailed literature review focused on existing markers for the species. This review was published in a report for the European project "Trees for Future" (http://www.trees4future.eu) whose aim is to provide genetic tools for future forest management. A modified version of this review is presented in chapter 2. Based on this and on a technical expertise on genetic marker development I had acquired before (Guichoux *et al.* 2011a; Guichoux *et al.* 2011b; Lefèvre *et al.* 2012), I started the design of nuclear microsatellites, as explained in chapter 3. This turned out to be, as in other conifers characterized by a large genome, a difficult and time-consuming technical challenge, for which I could take advantage of the development of next-generation sequencing techniques. These markers were eventually applied on a range-wide sample of 45 modern populations (24 individuals/population) and analysed in parallel with mitochondrial DNA data that had been produced by cooperation partners (S. Liepelt and co-workers, Marburg University). The aim was to identify and characterize recent translocations without any reference to fossil data (chapter 4).

The palaeoecological work also included an extensive literature survey to compile and correlate at the European scale larch fossil over the last 130,000 years. Based on this and on fossil data from different databases I reconstructed the history of larch including its responses to climate oscillations (short-term, long-term) and to anthropogenic influences without

making reference to the genetic data (chapter 5). In the final chapter 6, I synthesize fossil and genetic data to identify last glacial maximum refugia and to compare anthropogenic impacts identified by genetic tools versus by fossils. I also mention some ideas emerging from a related project dealing with larch canker, the most serious larch disease. Tolerance to this disease strongly differs between populations from different parts of the range, suggesting that recent translocations could have serious consequences if maladapted provenances are transplanted. A preliminary report of this project is provided in Appendix 1.

All chapters are conceptualized to allow an independent understanding.

Scientific contributions and acknowledgements

This study was funded by the German Research Foundation (DFG LI 582/18-1) and conceived as a bi-nationally supervised thesis. The paleoecological part was directed by Prof. Dr. Thomas Litt and carried out at the Steinmann Institute for Geology, Paleontology and Mineralogy of the University of Bonn (Germany). The population genetic part was supervised by Dr. Rémy Petit and Dr. Sophie Gerber and performed in the BIOGECO (Biodiversity, Genes and Communities) research unit affiliated to the University of Bordeaux and INRA (Institut National de la Recherche Agronomique). This allowed taking advantage of the expertises found at the two institutions, a precondition of this PhD. In addition, I collaborated with Dr. Sascha Liepelt and Prof. Dr. Birgit Ziegenhagen (University of Marburg, Germany) our partners in the DFG project.

In parallel I contributed to a project on larch canker that was directed by Dr. Dominique Piou and Dr. Cécile Robin. In addition, I had the possibility to discuss my research with Prof. Dr. Maria Fernanda Sánchez-Goñi, directing the laboratory of paleoclimatology and marine paleoenvironment affiliated to the EPHE (Ecole Pratique des Hautes Etudes) and integrated in the EPOC (Environnements et Paléoenvironnements Océaniques et Continentaux) research unit of the University of Bordeaux 1.

The chapters of this thesis represent the state of the art of my research and are at different stages of advancement: chapters 2 and 3 are published (http://www.trees4future.eu, Wagner *et al.* 2012). They appear in slightly modified versions in this thesis. Chapter 4 will be submitted after some final analyses are performed, whereas chapters 5 and 6 are preliminary reports. They will have to be significantly reworked before submission. All chapters benefitted from

the support of my supervisors and colleagues. In the following I document their contributions as an acknowledgement of their work and to clarify my own work.

Chapter 2

I compiled the literature and wrote the manuscript. It was revised by Rémy Petit. For comments and complementary information I thank Luc Pâques, Berthold Heinze and Barbara Fussi.

Chapter 3

I conceived the study, performed the experiments, produced and analyzed the data and wrote the manuscript. I got scientific advice during conception and writing from Rémy Petit and Sophie Gerber. Genotyping was performed in the Genome-Transcriptome facility of the Functional Genomic Centre of Bordeaux. I thank Franck Salin, Sarah Monllor, Christophe Boury, Philippe Chaumeil and Camille Lepoittevin as well as Andres Buser (*ecogenics GmbH*) for support and assistance during genotyping and primer design. For assistance in the various fields of this work I thank Vanina Guérin, Patrick Léger, Guillaume Lalanne-Tisné, Anne Laure Bouillot, Erwan Guichoux, Lélia Lagache, Olivier Lepais and Maxime Nardin. For support with plant material and assistance during sampling I thank Luc Pâques, Jutta Mäsgen, Laurent Bouffier, Frédéric Lagane, Christoph Sperisen, Yuri Hayda, Ihor Neyko, Guy Bour, Czeslaw Koziol, Philippe Rozenberg, Leopoldo Sanchez, Frédéric Millier, Jean-Paul Charpentier, Owe Martinsson, Lars Karlman, Hermann Spellmann (NW-FVA), Keiya Isoda, Nathalie Isabel, Marie-Claude Gros-Louis, the Ukrainian Research Institute for Moutain forestry, Ivano-Frankivsk, the Polish Osusznica Foerst Institute (Nadleśnictwo Osusznica), and the Polish Maskulińskie Forest Institute (Nadleśnictwo Maskulińskie).

Chapter 4

I conceived the study assisted by Rémy Petit. I performed the experiments and produced the nuclear data. Sascha Liepelt produced the mtDNA data. I analyzed the data and wrote successive drafts which I progressively improved with the help of Rémy Petit. I discussed the work at different stages with Sophie Gerber. Genotyping was performed in the Genome-Transcriptome facility of the Functional Genomic Centre of Bordeaux. I thank Franck Salin,

Sarah Monllor and Christophe Boury for support and assistance during genotyping and Vladimir Semerikov, Christina Huneck and Christina Mengel for their help in mitochondrial marker design. For assistance in various fields of the project I thank François Ehrenmann, Raphaël Leblois, Mathieu Gauthier, Vanina Guérin, Patrick Léger, Erwan Guichoux. For support with plant material see people and institutions acknowledged under chapter 3.

Chapter 5

I compiled and mapped the data and wrote the chapter. I improved it thanks to comments from Thomas Litt, Rémy Petit and Maria Fernanda Sánchez-Goñi. I thank Thomas Giesecke and collaborators, W.O. van der Knaap, Petr Kuneš, Rachid Cheddadi, Maria Fernanda Sánchez-Goñi for pollen database access. For essays in the application of vegetation models I thank Rachid Cheddadi, Manuel Chevalier, Christian Ohlwein and Sophie Stolzenberg. For support with regional literature and data I thank Brigitta Ammann, Elena Ortu, Jacques Louis de Beaulieu, Elisabetta Brugiapaglia, Eniko Magyari, Angelica Feurdean, Ion Tanţău, Elena Marinova, Vlasta Jankoska, Petr Pokorný, Ruth Drescher Schneider, Wojciech Granoszewski and Dariusz Krzyszkowski. For assistance in graphical issues I thank Annette Bohr.

Chapter 6

I wrote the chapter that I ameliorated thanks to comments from Rémy Petit.

Further acknowledgements

My special thanks go to my supervisors Thomas Litt, Rémy Petit and Sophie Gerber who motivated and supported my research. Merci beaucoup, vielen Dank, that each of you allowed me to take advantage of your individual expertise. Thank you Rémy, for the wealth of ideas and the huge amount of time you invested in this project. Thank you Sophie, for your critical questions and your straightness. Thank you Thomas, for starting this project and for giving the impetus to study such an exciting topic.

For additional funding I thank the DAAD (German academic exchange service), the UMR BIOGECO and the Paleobotany research group of the Steinmann Institute of the University of Bonn.

I thank Rachid Cheddadi, Martin Lascoux and Richard Pott for having accepted to write reports on this thesis, and Barbara Reichert for having accepted to be part of the examination committee.

Many thanks go to Maria Fernanda Sánchez Goñi, Raphaël Leblois, Rachid Cheddadi, Martin Lascoux for having been members of my PhD committee 2011 and for assisting afterwards. I thank Antoine Kremer for giving me scientific advice and motivating my research. For inspiring discussion I thank Stéphanie Desprat and Hans Jürgen Böhmer. For fruitful cooperation I thank Dominique Piou, Cécile Robin, Sascha Liepelt and Birgit Ziegenhagen.

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For constant encouragement and presence over long and short distance I thank my family and my friends. Herzlichen Dank an euch, Hedwig, Michael, Nadine, Richard, Annette, Laura, Mathis, Silvia, Frank, Jutta, Michael, Jolam, Anne, Gabi, Sascha, Marga und Beate. Merci beaucoup à vous, Frédo, Laurent, Vanina, Emanuel, Stéphanie, François, Sophie, Carol, Claire, Patrice, Olivier, Laure et Virgil. Herzlichen Dank an dich, Jürgen.

CHAPTER 2:

Description of the species and review of existing markers

Modified version of a

Trees4future contribution

http://www.trees4future.eu

Figure 1 *Larix decidua* in the Central Swiss Alps [photograph: C. Sperisen, 2010-10, with permission]

Species description

Larch (*Larix*) belongs to the Pinaceae family and is one of the most abundant coniferous genera of the northern hemisphere. The genus comprises 10 species occurring in Eurasia and North America (Farjon 2010). European larch (*Larix decidua*, Miller 1768) is endemic to Europe and is characterized by a strongly disjunctive distribution (Fig. 1, Fig. 2) (e.g. Bauer 2012; Geburek 2010; McComb 1955; Rubner 1953). The first and major continuous distribution area is located in the European Alps and is centred in the subalpine belt of the continental Central Alps but larch also occurs in other parts of the Alps over a wide altitudinal range. In the Western Alps, the upper limit is 2,300 m or even 2,900 m if one includes krummholz trees (i.e. small trees that do not grow into large canopy trees). The lower limit is 1,300 m in the south-western part of the Alps and 450 m in the north-western part. In the easternmost Alps the distribution is centred at lower altitudes with more humid climates. The upper limit in this part of the range is 1,600 m and the lower limit 300 m.

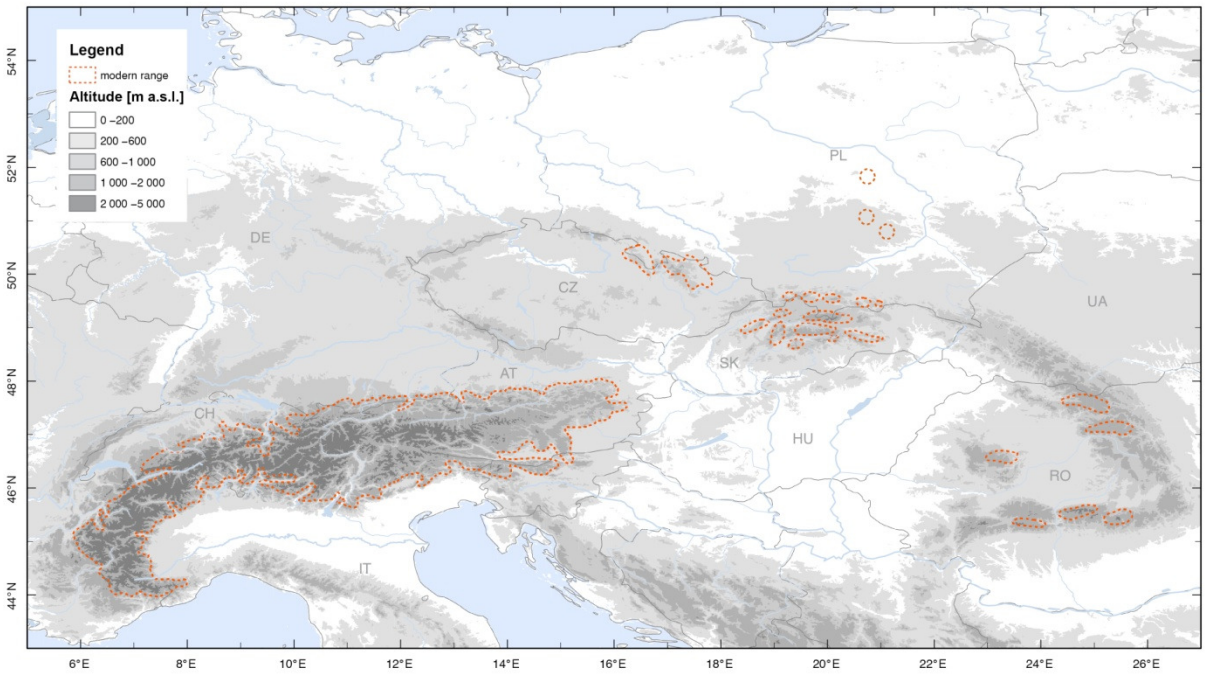

Figure 2 Distribution map of *Larix decidua* showing the current natural distribution. [Modern distribution data compiled by E. Welk, AG Chorology, Geobotany Department, University Halle, based on map 21b in Meusel *et al.* (1965); modified in central Poland]

European larch mainly grows in mixed stands associated with Norway spruce (*Picea abies*), stone pine (*Pinus cembra*), Swiss pine (*Pinus mugo*), European beech (*Fagus sylvatica*) or silver fir (*Abies alba*) but can also occur in pure stands. As a consequence of rural migration of populations towards the cities in the 19th century large areas of former grazing (upwards) and cultivation (downwards) lands have become recolonized or planted. The Bohemian, Moravian and Pannonian Plains separate the Alpine distribution part of the range from the Carpathian one, which is split into four sub-regions. The first area is the Eastern Sudety Mountains where larch mainly grows between 300 and 800 m together with Norway spruce, silver fir and European beech. The second one is the Polish lowland with main occurrences between the rivers Oder and Weichsel at an altitude ranging from 160 m to 600 m. The third is the Western Carpathian area with major occurrences in the Tatra Mountains (High Tatra 1,100-1,300 m, Low Tatra 600-1,000 m) and in the bordering area of the Beskids, Fatra and Ore Mountains. Associated species are Norway spruce, stone pine, European beech and Scots pine. The fourth area comprises small and highly disjunctive stands in the Eastern Carpathians (1,200-1,800 m) and Southern Carpathians (650-1,900 m) and in the Bihar Mountains, together with Swiss pine and Norway spruce.

Morphologically and ecophysiologically differentiated ecotypes are found in each of the areas described above. These ecotypes were previously treated as subspecies of *L. decidua* (ssp. *decidua*, ssp. *sudetica*, ssp. *carpathica* and ssp. *polonica*). Farjon (2010) identifies only three varieties (var. *decidua*, var. *carpathica*, and var. *polonica*). A crucial result of a multi-site *L. decidua* provenance trial was the finding that Central European provenances, in particular the Sudety ones as well as alpine provenances from lower altitudes, are less canker sensitive than the other alpine provenances, especially the western Alpine provenances (Schober 1977; Schober 1985). Larch canker is transmitted by the ascomycete *Lachnellulla willkomii* (Hartig) Dennis (Schober 1949; Willkomm 1867) and is the most serious of European larch diseases.

Delimitation of the natural range of European larch is very challenging. This is caused by a long history of planting, starting in the 16th century and culminating in the 19th century (e.g. Rubner 1953; Schober 1949; Tschermak 1935). During this time, larch was extensively planted within and beyond its natural range, leading to uncertainties about the autochthony of populations, in particular in the Central European region. An unexpected attendant phenomenon of these extensive plantations was the expansion of larch canker, which contributed in a major way to the decrease of plantations in the 20th century. Later on these problems were attributed to the use of canker-sensitive material in afforestation (e.g. Schober 1977). Japanese larch (*Larix kaempferi* (Lamb.) Carr.) and hybrids of European and Japanese larch (*Larix* × *eurolepis* Henry) have also been planted in Europe, but only since the middle of the 20th century. The hybrid combines favourable properties of the parental species such as stem straightness, drought-tolerance and low canker sensitivity (Acheré *et al.* 2004; Bauer 2012; Geburek 2010). European larch is a monoecious, diploid (2n=24), mainly outcrossing, deciduous pioneer tree, which becomes mature after 20-30 years (Dieckert 1962; Geburek 2010). Flowering period is from (February) March to April (May). Its wind-dispersed pollen does not have air sacs like the pollen of other conifers, resulting in less extensive dispersal (Sjögren et al. 2010; Sjögren et al. 2008). Seeds are ripe in autumn of the flowering year. They are enclosed in cones within winged nutlets. Cones stay on the tree over 2-3 (5) years and shed seeds under dry weather conditions. Seeds are mostly wind-dispersed and mast seeding years occur approximately every 10 years in this species (Rameau *et al.* 1993). Clonal propagation resulting in small clusters of clonal trees can occur when sapling branches touch the ground and get rooted (Pluess 2011). Larch is a light-demanding pioneer tree with a low competitive ability. It is outcompeted by other species in climax stages whereas its persistence is favoured in different kinds of disturbance-driven ecosystems. Under optimal conditions and

free standing it can reach maximum ages of 600-850 years. Under forest conditions, it reaches only about 150 years (Bauer 2012; Geburek 2010). Even though European larch is currently less planted than some other conifers such as Norway spruce or Douglas-fir, it is still very much appreciated because of its fast juvenile growth, low sensitivity to most diseases as well as to wind, snow, fire, air pollution and chemicals. Its wood is characterized by a high density, long durability, good elasto-mechanical properties, and good aesthetic value. For these and for other reasons it is used for afforestation, reforestation and as construction-timber for outdoor and indoor equipments or barrels for conservation of chemicals. Examples illustrating its excellent construction properties are the world's highest wooden tower (118 m, Gliwice, Poland) and several Venise bridges (De Miranda *et al.* 2010; Grosser & Ehmcke 2012).

Concerning the material to be traced it would be of great interest to have markers allowing to elucidate populations' status (native/non-native) and to evaluate the potential effects of introgression (inter- or intraspecific), in particular in regions that have been strongly impacted by recent introductions. This would allow a better delimitation of its natural range. Second, it would be important to trace material used in breeding populations and in forest reproductive material (FRM) to ensure the use of only well performing provenances and varieties for plantation. Third, plantations showing unexpectedly high canker sensitivity should be studied to clarify their origin. Another type of material that could be profitably analysed is wood logs of unknown origin to fight illegal logging.

Review of existing genetic markers

For some reason marker development in European larch has been lagging behind that observed in other European tree species, although its three differently inherited genomes (nuclear: biparentally inherited, mitochondria: maternally inherited, chloroplast: paternally inherited) offer ideal preconditions for powerful marker combinations. Genetic studies started about 40 years ago with the development of isozyme markers (Mejnartowicz & Bergmann 1975). Isozyme markers are still used today (Konnert & Behm 2006; Müller-Starck & Felber 2010). Other nuclear and organelle markers have primarily been designed for the genus to address issues at the interspecific level (e.g. Acheré *et al.* 2004; Gros-Louis *et al.* 2005; Semerikov & Lascoux 2003). Only very recently nuclear species-specific SNP markers have been developed (Mosca *et al.* 2012b). Other markers (oleoresins) have been tested with some

success. They allowed the distinction between Alpine and other provenances and between European larch from Japanese larch as well as from hybrids of European and Japanese larch (Lang 1976; Weissmann & Reck 1987).

Material analysed, DNA extraction and fragment analysis

DNA has been successfully isolated from various tissues such as megagametophytes (e.g. Mosca *et al.* 2012b), seedlings (e.g. Semerikov & Lascoux 2003), leaves (e.g. Pluess 2011), buds (Scheepers *et al.* 2000) and embryogenic mass (L. Pâques, pers. comm.). For isozyme analyses megagametophytes have been commonly used (e.g. Lewandowski & Mejnartowicz 1991a). DNA isolation mostly followed CTAB (Devey *et al.* 1996; Doyle & Doyle 1990) or manufactured isolation kits such as DNeasy Plant Mini Kit (Qiagen, Gros-Louis *et al.* 2005) or DNAeasy 96 Plant kit (Pluess 2011).

Organelle markers (mitochondrial and chloroplast DNA)

Organelle markers applicable on *Larix decidua* have been developed for questions such as phylogeny and species diagnostics. Chloroplast DNA variation has been studied based on sequence variation (Gros-Louis *et al.* 2005; Qian *et al.* 1995; Wei & Wang 2003), PCR-RFLPs and microsatellites (Semerikov and Lascoux 2003; Acheré et al. 2004). Mitochondrial DNA variation has been studied using direct PCR, PCR-RFLPs (Acheré *et al.* 2004; Semerikov & Lascoux 2003; Semerikov & Polezhaeva 2007) and sequencing (Gros-Louis *et al.* 2005). These studies have identified useful markers and marker combinations for species discrimination. By combining a mitochondrial and a chloroplast marker, *L. decidua* and *L. kaempferi* could be discriminated (Acheré *et al.* 2004; Jagielska 2008). In another study, *L. decidua* and *L. kaempferi* could be discriminated from *L. sibirica* and *L. laricina* by sequencing four chloroplast regions (*matK, trnL-intron*, *trnT-trnL trnL-trnF*) and all four species could be identified by sequencing five mitochondrial introns (*cox1-1*, *matR-1*, *nad1-b/c*, *nad3-1* and *nad5-1*; Gros-Louis *et al.* 2005). Note that in the same study Gros-Louis *et al*. (2005) also obtained nuclear markers discriminating the four species (see nuclear markers section below).

Nuclear markers

AFLPs (amplified fragment length polymorphisms)

Arcade *et al.* (2000) constructed single-tree genetic linkage maps of European and Japanese larch using 114 AFLPs resulting from 5 AFLP primer combinations. Note that in the same study they also used 149 RAPD and 3 ISSR loci. AFLPs were also used in phylogenetic reconstruction of the genus *Larix* (Semerikov *et al.* 2003). In the latter case the authors applied six primer combinations and scored 442 polymorphic fragments in 11 species.

Isozymes

Until 2011 isozymes have been the only nuclear markers used to study intraspecific variation in European larch. A common set of 13 isozyme markers is used in the German Züf certification system to trace forest reproductive material (Konnert & Behm 2006). First attempts for studying intraspecific genetic variation across the range have been made by Lewandowski and Mejnartowicz (1991a) and Maier (1992). These studies led to some valuable information but as marker resolution is limited and sampling was not representative (both studies relied on seven populations), further investigations are necessary. One suggestion coming from Maier's study is that populations from the Eastern Alps are genetically closer to the Central European ones than to the Western Alpine ones. On the regional scale, a study in the Western Alps region of Piedmont found that larch populations from the Western Alps (Torino) and the Italian Central Alps (Verbania) form a common genetic group whereas a population from the southernmost Maritime Alps (southern Cuneo) was clearly different (Belletti et al. 1997). However, sampling was uneven and should be refined to confirm the results. Another study included 26 populations from across the Alps and showed that there is no important loss of genetic diversity between old stands and their natural regeneration. It further revealed that genetic variation in *L. decidua* is lower than in *Picea abies* and *Pinus mugo* but higher than in *Abies alba* (Müller-Starck & Felber 2010; Müller-Starck et al. 2000). Finally, a study carried out in Romania highlighted genetic peculiarities and relationships of the five fragmented Romanian centres of natural larch occurrence (Mihai & Teodosiu 2009).

Microsatellites

Microsatellites in larch have been developed for different larch species (Chen *et al.* 2009; Isoda & Watanabe 2006; Khasa *et al.* 2006; Khasa *et al.* 2000). Microsatellite development for European larch has been performed in this study (see Chapter 3).

SNP

SNPs have first been used in larch in a study aiming at distinguishing species (i.e. *L. decidua, L. sibirica, L. kaempferi* and *L. laricina*; Gros-Louis *et al.* 2005). They resulted in the identification of three gene loci with fixed interspecific polymorphisms implicating 17 SNPs and two indels. Recently Mosca *et al.* (2012b) used a candidate gene approach to study nucleotide diversity and genes departing from neutral expectation in the four alpine species *L. decidua, Pinus cembra, Pinus mugo* and *Abies alba*. 800 genes originally sequenced in *Pinus taeda* were resequenced in each the four species, resulting in 307 SNPs for *L. decidua.* This study was rather exploratory for larch as sequencing success was relatively low and sampling was not representative. A subsequent study dealt with geographical and environmental determinants of genetic diversity of the four alpine conifer species mentioned above (Mosca *et al.* 2012a). For *L. decidua* this was based on 267 SNPs discovered in the first study and revealed three genetic clusters across the Alps.

ITS

ITS sequencing of ITS1, the 5.8S ribosomal RNA gene and ITS2 was used in phylogenetic reconstruction of the genus *Larix* (Gernandt & Liston 1999; Semerikov *et al.* 2003). ITS and AFLP phylogenies were very similar and reveal a basal position of *L. decidua* in the clade of Eurasian larch species.

ISSR

Three ISSR (inter-SSR) regions were used in combination with RADPs and AFLPs for genetic mapping of European and Japanese larch (Arcade *et al.* 2000).

RAPD

RAPD fingerprints were used to discriminate *L. decidua*, *L. kaempferi* and their hybrid *L. x eurolepis* to investigate hybrid performance (Arcade *et al.* 1996; Scheepers *et al.* 2000). Furthermore they were used in combination with AFLP and ISSR for genetic mapping of European and Japanese larch (Arcade *et al.* 2000).

Levels of differentiation

Phylogeny, evolutionary history, distinction of species, subspecies and hybrids

The phylogeny of the genus *Larix* has been reconstructed using various molecular markers (Gernandt & Liston 1999; Qian *et al.* 1995; Semerikov *et al.* 2003). All studies showed three monophyletic clades: a North American clade, a South Asian clade and a North Eurasian clade, with *L. decidua* having a basal position in the Eurasian clade. Special attention has been paid to the var. *polonica* that has been hypothesized to be a hybrid of *L. decidua* and *L. sibirica* larch (Bobrov 1972). Hybrids of European and Japanese larch (*L.* x *eurolepis*) have been artificially introduced in the 20th century.

To date conclusions about levels of genetic differentiation in *Larix decidua* have been drawn from studies focusing on the local and regional scales. On a very local scale (<5km) Pluess (2011) has reported an F_{ST} value of 0.014 based on nuclear SSR data. On a broader scale (distance between the populations up to 200km), Mosca *et al.* (2012a) have obtained an F_{ST} value of 0.011 based on nuclear SNP data. Finally, at the scale of the Italian Alpine region (populations located up to 600 km from each other), Mosca *et al.* (2012a) have reported a mean F_{ST} of 0.04, indicating a higher degree of differentiation that can be explained by the subdivision of the studied populations in three major genetic groups, as revealed by Bayesian cluster analysis.

Glacial refugia, biogeographic history

So far, biogeographic history of European larch has not been studied in detail as it is the case for the other European tree species e.g. *Quercus* (Petit *et al.* 2002), *Fagus sylvatica* (Magri *et al.* 2006) and *Pinus sylvestris* (Cheddadi *et al.* 2006) A detailed reconstruction of its history

of the last 130,000 years is done in this study using newly compiled fossils and genetic data (see Chapter 4 and Chapter 5).

Stand, seed and pollen dispersal, small scale genetic structure

Pluess (2011) has performed a landscape-scale analysis of *L. decidua* along the lateral moraine and the adjacent valley slope of a glacier in the Swiss Alps at 1700–2240 m a.s.l.. Nine SSR markers were used for this purpose. All sampled individuals (N = 730) formed a single genetic cluster indicating homogenizing gene flow despite spatial genetic structure (SGS) up to 80 m. No evidence for selfing or for inbreeding was found in adults or in juveniles (heterozygote deficit was not significantly different from zero). SGS among juveniles was found at up to 30 m in the older sub-population whereas no SGS was found in the younger, recently established sub-population. A maximum likelihood paternity assignment revealed local gene dispersal in the ancient part (2–48 m) and intermediate-to-long distance dispersal into the recently colonized part (115–3132 m), pointing to intensive mixing of the genes in this expanding population and suggesting that genetic diversity can be maintained during rapid population expansion driven by climate warming.

Individual tree, parent trees

Genetic distances among European and Japanese larch clones were estimated based on RAPD markers to investigate the link between heterozygosity and heterosis in hybrid larch (Arcade *et al.* 1996).

Use of a traceability system

Currently 13 isozyme loci are used for European larch in the German ZüF (Zertifizierungsring für überprüfbare Forstliche Herkunft Süddeutschland e.V.) certification system (Konnert & Behm 2006).

Traceability systems in use and future needs

Species identification

In European larch several markers have been tested for species identification. First, there is a combination of one chloroplast and one mitochondrial marker that can discriminate *L. decidua* and *L. kaempferi* and determine the parental species of their hybrid *L.* x *eurolepis*. This approach is more powerful than isozymes and generally results are satisfying. However, there is the disadvantage that chloroplast DNA analysis is based on the presence (*L. kaempferi*) or absence (*L. decidua*) of DNA fragments, which in some cases leads to ambiguous results (Acheré *et al.* 2004; Jagielska 2008). The use of pure species parents controls partly solves this problem. Second, sequencing organelle and nuclear markers can discriminate the four species *L. decidua, L. kaempferi, L. sibirica* and *L. laricina* (Gros-Louis *et al.* 2005; cf. Organelle marker section). Moreover infrared spectroscopy (NIRS) of needles and wood is currently tested as an alternative (L. Pâques pers. comm.). A future task will be to evaluate discrimination power of the microsatellites developed in this study for identification of different species as well as advanced hybrid generations. This is particularly needed as future seed orchards will frequently work with material from up to three hybrid generations including backcrosses.

Identification of the origin of FRM

The only method to identify the origin of seedlings declared in certification documents is to compare the seedlings to a reference sample. In Germany such a comparison is performed with the Züf System. An *ad hoc* assignment of FRM to its provenance region would be crucial for future silviculture preventing the choice of maladapted provenances. The markers and the range-wide reference sample of this study could be used for attempts in this direction (see Chapter 4).

Inference of the number of mother trees from a seed lot by molecular methods

Genetically improved seed lots from clonal seed orchards could be controlled with highly polymorphic markers using parentage analyses. This would allow tracing this material to the seed orchards where it was produced (or possibly ruling out such an origin) and enumerating the number of parental clones involved in the composition of each seed lot. The only

reference needed would be one representative of each clone. In principle, such an approach could also be used on unknown seed lots, although more developments would be necessary.

Clone identification, including clonal mixtures

Other than for hybrids of European and Japanese larch there are to our knowledge no commercial plantations with clonal material of European larch. However, clones are used in seed orchards to produce inter- and intraspecific hybrid seeds. Clonal identification with the help of sufficiently variable nuclear markers could help assessing the exact composition of seed orchards to control propagation operations.

Identification of introgression

Identification of introgression would be an important topic to address in regions where exotic provenances of European larch or Japanese larch have been detected.

Wood identification in relation to illegal logging

Illegal logging of old growth larch forests in Siberia is a topic of concern. Developing methods to trace this material with DNA isolated from logs (cambium or wood) would be of high relevance. However *L. sibirica* is also planted in some European countries makes it more complicated. Nevertheless, checking the conformity of the logs to the species could be a first useful step, as in Siberian forests several larch species occur.

Conclusions

Marker development and studies of European larch are not very numerous and as a consequence knowledge about this species has stayed limited. Powerful markers (SNPs, SSR) are urgently needed to open new perspectives for a number of practical applications in forest research and in forest management. They should enable precise monitoring of material produced in seed orchards as well as the identification of native and introduced material from across the range. This should guide plantation forestry and help focus conservation efforts in a species with a particularly intense history of provenance translocations.

Supporting Information

Table 1: Organelle markers used in *Larix decidua*

Fragment	Type	Analysis method	Spatial scale	Scope	Reference
TF	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
rpl20trnW	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
rpl20trnW	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
rpl20trnW	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al*. 2003
trnLV	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
CS	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al*. 2003
psbD	Chloroplast	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
Pt9383	Chloroplast	Microsatellite	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al*. 2003
Pt26081	Chloroplast	Microsatellite	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
Pt30204	Chloroplast	Microsatellite	Northern hemisphere	*Larix* phylogeny	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
ll	Chloroplast	PCR RFLP	Eurasia *L. decidua, L.kaempferi*	Species discrimination	Acheré *et al.* 2004
trnT-trnF	Chloroplast	Sequence	Northern hemisphere	*Larix* phylogeny	Wei and Wang 2003
matK	Chloroplast	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
trnL	Chloroplast	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al*. 2005
trnT-trnL	Chloroplast	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
trnL-trnF	Chloroplast	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al*. 2005
nad5-1/2	Mitochondrial	PCR RFLP	Northern hemisphere	Larix phylogeny Postglacial history Siberia	Semerikov & Lascoux 2003, Semerikov *et al.* 2003
nad4-3c/4r	Mitochondrial	PCR RFLP	Northern hemisphere	*Larix* phylogeny Postglacial history Siberia	Semerikov & Lascoux 2003, Semerikov *et al.* 2003

Fragment	Type	Analysis method	Spatial scale	Scope	Reference
UBC460	Mitochondrial	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov *et al*. 2006
				Postglacial history Siberia	Semerikov *et al.* 2007
R11	Mitochondrial	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov *et al*. 2006
				Postglacial history Siberia	Semerikov *et al*. 2007
C8	Mitochondrial	PCR RFLP	Northern hemisphere	*Larix* phylogeny	Semerikov *et al.* 2006
				Postglacial history Siberia	Semerikov *et al.* 2007
F13	Mitochondrial	Direct PCR	Eurasia *L. decidua, L.kaempferi*	Species discrimination	Acheré *et al*. 2004
Cox1-1	Mitochondrial	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
matR-1	Mitochondrial	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
nad1-b/c	Mitochondrial	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
nad3-1	Mitochondrial	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al.* 2005
nad5-1	Mitochondrial	Sequence	Northern hemisphere *L. decidua, L.kaempferi L. sibirica, L. laricina*	Species discrimination	Gros-Louis *et al*. 2005

CHAPTER 3:

Two highly informative dinucleotide SSR multiplexes for European larch

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Resources

Abstract

We have designed two highly polymorphic microsatellite multiplexes for *Larix decidua* Mill. (European larch), a coniferous tree species with a fragmented distribution across Europe. The multiplexes combine microsatellites previously designed for the sister species *L. kaempferi* and newly identified microsatellites obtained by pyrosequencing of an enriched microsatellite library and subsequent marker candidate selection. As we wanted to target highly polymorphic markers, only microsatellite motifs with a high number of repeats (≥12) were selected. An important proportion of the marker candidates presented multiple bands, bad amplification or insufficient polymorphism. Such difficulties were expected owing to the large genome size of the studied species. Our strategy for marker validation followed most recent recommendations for microsatellite development, e.g. verifying marker quality in terms of polymorphism and accurate allele binning before multiplexing. The most promising loci were combined in two multiplexes, a 7-plex and a 6-plex. These were tested on a sample of 413 individuals from 18 populations distributed across the natural range. The 13 loci had from 9 to 36 alleles. Markers were successfully tested in another laboratory, confirming robustness of the marker protocols. We also tested transferability on six other larch species from Asia and North America. Overall, this study shows that, even in species with large genome size and relatively low overall polymorphism, microsatellites can be successfully developed using next-generation sequencing technologies provided that some additional precautions are taken compared to species lacking these characteristics.

Introduction

Larix decidua is an endemic European conifer with a highly fragmented montane to subalpine distribution in the Alps, the Sudety, the Tatra and the Carpathians as well as some exceptional lowland occurrences in Poland (McComb 1955; Rubner 1953). As these regions, especially the mountainous ones (e.g. Colombaroli *et al.* 2010; Tinner & Kaltenrieder 2005), are strongly exposed to climate change, there is an urgent need for detailed range-wide genetic studies that can provide information for preservation of valuable genetic resources and sustainable forest management. So far, range-wide genetic studies based on nuclear markers in *L. decidua* have exclusively relied on allozyme markers. Such studies have yielded useful information about genetic relationships among populations (Lewandowski *et al.* 1991; Lewandowski & Mejnartowicz 1991a, b; Maier 1992). However, to better understand past population dynamics of the species an increased resolution is needed. We have therefore

endeavoured to develop highly polymorphic nuclear microsatellites. The development was based on the transfer of existing markers from sister species and the selection and design of new markers based on 454 pyrosequencing. First, we tested existing markers from the Asian sister species *L. kaempferi* (Isoda & Watanabe 2006) and the North American species *L. occidentalis* (Chen *et al.* 2009; Khasa *et al.* 2000). As this did not lead to a satisfactory number of markers matching recommended quality criteria, e.g. sufficient polymorphism and clear binning of alleles (Guichoux *et al.* 2011a), we developed new markers based on pyrosequencing of an enriched microsatellite library. This approach can be substantially more cost-effective as the conventional method based on the screening of cloned libraries by Sanger sequencing (e.g. Santana *et al.* 2009). However, in our study, we had to face two difficulties. First, initial tests had shown that markers with <12 repeats (i.e. for the individual which was initially sequenced) were characterized by low variation. This low variation might in part be attributed to the fact that trees and shrubs, which have relatively long generation times, generally show lower rates of molecular evolution than related herbaceous plants (Smith & Donoghue 2008). To augment the chances to find variable markers we focused on microsatellites with a high number of repeats (≥12) as microsatellite polymorphism is known to increase exponentially with the number of microsatellite repeats (Ellgren 2000; Kelkar *et al.* 2008). Second, in coniferous species with large genomes such as *L. decidua* (11,198 Mb, Greibhuber 1986), a large proportion of microsatellites can be expected to be located within repetitive DNA sequences, leading to marker candidates that are difficult to amplify (e.g. Pfeiffer *et al.* 1997). We therefore targeted a large number of marker candidates to select those showing both clear amplification profiles and sufficient polymorphism. Finally, we decided to combine the markers in multiplex reactions, i.e. PCR reactions amplifying several markers simultaneously, because once such multiplexes are established they enormously reduce laboratory costs and labour time and thus enable high throughput analyses and promote accuracy and precision of the genetic result (Guichoux *et al.* 2011a; Guichoux *et al.* 2011b; Lefèvre *et al.* 2012). By following all these steps, we obtained two highly variable multiplex for *Larix decidua* (one 7-plex and one 6-plex).

Materials and methods

Plant material and DNA isolation

In 2010, we collected phloem and needle samples from 18 populations forming a gradient over the natural distribution range (Table 2). Eight had been collected *in situ* and 10 *ex situ* in four German provenance trials. In the latter case, each population sample (consisting in 24 individuals) originated from one single trial. Further samples for marker validation as well as for transferability tests were provided by colleagues. These included six progenies (each comprising one female parent and seven offspring) that were collected in a progeny trial (Planches, France). Note that progeny tests in this study only give a rough insight into Mendelian segregation but should help detect null alleles. Originally we planned to start with 12 progenies (12 female parents and 7 offspring) based on seeds. Due to problems with material, we were had to work with the six progenies described above. Furthermore, we obtained DNA samples of another six *Larix* species. These were *L. sibirica* (21 individuals from one population), *L. kaempferi* (12 individuals from 12 populations) and *L. gmelinii* var. japonica (12 individuals from 12 populations), all from Eurasia. From North America, we obtained samples from *L. laricina* (10 individuals from 10 populations), *L. lyallii* (4 individuals from 4 populations), and *L. occidentalis* (4 individuals from 4 populations). For the samples that we collected ourselves, we mostly relied on phloem as tree height (up to 40m in the trials) made it difficult to collect needles. Phloem was sampled by using a hammer and small leather punch (Ø=1cm, length=10cm). The sampling technique we developed was rapid and easy. The leather punch was positioned between the bark scales (which can be very thick) and with one to three slight hammer strokes a small but sufficient sample (Ø=1cm, depth 1.5cm) was recovered. Damage to the tree was minimal and fast regeneration was ensured by sampling during the growing season. Samples were then put into tea bags that were stored in sealed plastic bags with 10g of silica gel. We isolated DNA from all individuals using 96-well plates. Starting material was mostly phloem (1cm disc 0.5mm thick), but in some cases needles were used (1-3 needles, cut into 2mm pieces). For material disruption, we added two 4mm-tungsten beads to the wells with the starting material. The plates were frozen during 1min in liquid nitrogen before a 1:30 min disruption by a Mixer Mill (Retsch, Germany). This step was repeated once. An Invisorb DNA 96 plant HTS kit (Invitek, Germany) was then used for DNA isolation following the manufacturer protocol. After isolation, DNA quality was evaluated on a 1% (w/v) agarose gel stained with GelRed (Biotium, USA).

Table 2 Geographical origin of the 18 *Larix decidua* populations for SSR design

Pop ID	Provenance	Country	Lat	Long	Altitude	Trial name	Nb analyzed individuals
4	Fernpass	Austria	47.37	10.90	1150	Münden	24
8	Semmering	Austria	47.63	15.77	1200	Riedesel	20
9	Lammerau	Austria	48.11	15.93	610	Münden	21
10	Neulengbach	Austria	48.05	15.93	560	Winnefeld	21
21	Pragelato	Italy	45.02	4.93	1900	Riedesel	22
24	Briançon/Montgenèvre	France	44.93	6.72	1730	Riedesel	22
39	Zabřeh-Dubicko	Czech Republic	49.83	16.97	400	Riedesel	24
40	Ruda	Czech Republic	49.98	16.90	480	Riedesel	24
42	Góra Chełmova	Poland	50.80	21.10	347	Sellhorn	24
43	Bliźyn	Poland	51.07	20.73	330	Sellhorn	24
72	Rekowo	Poland	54.08	17.46	184	*in situ*	20
73	Ruciane Nida	Poland	53.64	21.54	132	*in situ*	24
79	Brusturjany	Ukraine	48.42	24.02	1100	*in situ*	24
81	Vallée de la Tinée	France	44.09	7.09	1070	*in situ*	24
82	Sils Maria	Switzerland	46.43	9.77	2000	*in situ*	24
83	Zinal	Switzerland	46.11	7.64	2000	*in situ*	23
84	Sinaia Forest	Romania	45.33	25.50	1500	*in situ*	24
85	Voineasa Forest	Romania	45.37	23.93	1000	*in situ*	24

DNA concentration was determined by an eight channel Nanodrop spectrometer and adjusted to 10ng/µl on a STARTlet 8 channel robot (Hamilton, USA).

Multiplex optimization

Multiplex optimization followed the recommendations given in Guichoux et al. (2011a) to guarantee high marker quality, sufficient polymorphism and unambiguous allele binning. Screening for marker candidates started by a literature search of available markers from the closest sister species *Larix kaempferi* (Isoda & Watanabe 2006) and the North American species *L. occidentalis* (Chen *et al.* 2009; Khasa *et al.* 2000). These were tested in simplex reactions. As this did not lead to a sufficient number of suitable candidates, we designed new markers based on 454 sequencing of an enriched microsatellite library. Sequencing was done by the Swiss company ecogenics GmbH. In this sequencing approach, fragments were selected according to their size from genomic DNA enriched with simple sequence repeat (SSR) motifs by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The SSR enriched library was analyzed on a Roche 454 platform using the GS FLX titanium reagents. We worked on 1/16th of a 454 run. For the selection of candidate

markers, we used as main criterion the number of dinucleotide repeats (≥12). Note that this decision was taken after a failed attempt based on 454 sequencing of another enriched library produced by another company (data not shown). The problem had been that most of the marker candidates had had less than 12 repeats and were monomorphic or displayed limited variation (≤4 alleles). Those having 12 or more repeats (15 out of 100) had showed bad amplification or multiple bands. Hence, this experiment was abandoned. The main reason for the failure of the first experiment seemed to be poor sequencing quality and insufficient read length, making it difficult to design primers in the flanking regions of microsatellites with the targeted repeat unit number. In the second attempt, more candidates were obtained. Polymorphism and profile quality were first checked in simplex reactions using the M13-technique (Schuelke 2000). The PCR products were separated on a capillary sequencer (ABI-3730, Applied Biosystems, USA). Each marker was tested on seven individuals from across the range and on an additional DNA pool composed of 12 individuals from different geographic origins. Markers with low polymorphism (<5 alleles/7 individuals, unless additional alleles could be found in the pooled DNA sample) and profiles of low quality (multiple bands, bad amplification) were discarded. Second, the remaining candidates were tested for the presence of null alleles and for large allele dropout (e.g. non amplification of the longer allele due to differential amplification success) by using segregation analyses. For this purpose, six families composed of the female parent and seven offspring were screened. Third, based on observations of optimal annealing temperature for the markers and by taking into account the size range of each locus, we composed the two multiplexes. For the multiplex reaction, we used the Qiagen Multiplex PCR kit (Qiagen, Germany). Final volume and final concentration of Mastermix were optimized to reduce final costs (Guichoux *et al.* 2011a). PCR mix for both multiplexes was composed of 4.75µl of sterile water, 4µl of Qiagen Multiplex Buffer (2×), 1.25µl of primer premix and 3µl of DNA (10ng/µl). Concentrations of the primer pairs in the primer premix are given in Table 2. The cycling conditions for the two multiplexes differed only in the number of PCR cycles and were as follows: an initial step at 95°C for 15min; then 35 (multiplex1)/30 (multiplex 2) cycles at 94°C for 30s, 56°C for 1min and 72°C for 60s; and a final incubation at 60°C for 30min. PCR products were separated on 3% agarose gels stained with GelRED (Biotium, USA) and diluted 18 times in pure water for genotyping with the internal lane size standard LIZ600 on an ABI 3730 (Applied Biosystems, USA). This optimized protocol was communicated to a collaborating laboratory (INRA Orléans, France) to test its robustness. We also tested transferability on the six other larch species from Asia and North America.

Genotype scoring and analyses

For subsequent genotype scoring and accurate allele binning based on raw sizes (Guichoux *et al.* 2011a), we used STRand (http://www.vgl.ucdavis.edu/informatics/download_strand.php) and Autobin (http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin). For each marker, clear reading rules were defined and illustrated in a handout provided to all readers to increase reading consistency. A first estimate of error rate was obtained by counting mismatches on the basis of six positive controls repeated five times and of 10 randomly repeated controls, leading to 34 repetitions (8% of the complete data set). It should be emphasized that positive controls are important to include right from the start as they allow verifying differences across sequencing runs of a given laboratory but also, importantly, across different laboratories. This enables accurate and easy data exchange and combined data analyses. A second estimate of error rate was obtained by comparing scoring across readers at a subset of 264 individuals. Two types of errors were distinguished. Type A corresponds to cases where reader 1 calls a genotype as heterozygous and reader 2 as homozygous or vice versa. Type B corresponds to cases where a wrong allele is called by one of the readers. These error types can be corrected after being identified. There can be other kinds of incoherencies between readers, for instance if reader 1 calls a genotype and reader 2 sets missing data or if readers are calling different genotypes due to profile ambiguities which, in contrast to type A and B errors, cannot be corrected and eventually have to be set as missing data. Total allele number (nA), observed (H_O) and expected heterozygosity (H_E), inbreeding index (F_{IS}) and fixation index (F_{ST}) were estimated based on 413 individuals from 18 populations from across the range with 20-24 individuals per population (Table 2) using GENEPOP (Raymond & Rousset 1995; Rousset 2008) and GENEALEX (Peakall & Smouse 2006). Presence of null alleles was checked on a subset of 12 populations that had a minimum of 19 individuals without missing data using MICRO-CHECKER v.2.2.0.3 (Van Oosterhout *et al.* 2006).

Results and Discussion

Multiplex optimization

We tested the nine most promising candidate loci originating from the literature in simplex reactions. Most promising means that these loci had already been proven to amplify well and to be polymorphic (Pluess 2011). The 454 sequencing approach resulted in 27,041 reads with an average length of 184 base pairs. Of these, 3,311 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Primer design was possible in 312 reads, of which 100 were tested. Simplex PCR conditions had to be adjusted for each marker. This was because some candidates with long motifs needed more PCR cycles than others to be successfully amplified whereas such an increase resulted in non-specific products for other candidates. Testing marker profile quality and polymorphism (with a target of ≥5 alleles) over seven individuals and an additional DNA pool resulted in the selection of seven out of nine loci originating from the literature and 26 out of the 100 newly designed candidates. For identifying the latter, we started by excluding 34 markers with stutter bands or bad amplification, as identified on agarose gels. After genotyping the 66 remaining candidates with ABI capillary sequencer, we removed loci with multiple bands (19 markers), bad amplification (9 markers) or insufficient polymorphism (12 markers), leaving only 26 suitable markers. Low success rates have also been reported in other plant species with large genomes (e.g. Pfeiffer et al. 1997; Röder et al. 1995). In particular, a detailed investigation of the causes of amplification failures of microsatellite markers in *Picea abies* using Southern blot experiments showed that, in six out of seven cases tested, loci showing high quality profiles corresponded to single or low-copy sequences (Pfeiffer *et al.* 1997). This suggests that to identify sufficient candidate loci in species with large genomes, careful evaluation of the sequencing depth is necessary. Indeed, many markers will fail to produce clear PCR if the flanking regions (used to design the primers) are repeated elsewhere in the genome.

The other evaluation steps included allele binning as well as tests of Mendelian segregation to search for large allele dropout or null alleles. This led to the selection of a subset of 22 candidates for multiplexing for which binning was clear. There was no indication for non-amplification corresponding to large allele dropout. Null alleles were apparent at some markers (Ld50, bcLK189, bcLk263, Ld42, Ld45, Ld30, Table 3 and Table S1, Supporting Information). They were identified by comparing the female parent genotype with

Table 3 Characteristics of multiplexes 1 and 2 based on 413 *Larix decidua* samples from across its distribution range

Locus*	Reference	Primer sequences (5´-3´)	Motif	Dye	[C][1]	Size (bp)	N_A	H_O	H_E	F_{IS}	F_{ST}	*mism*†
Multiplex 1												
Ld31	this study	F: TTGAACTAGGGAGATCCGGC	$(AC)_{18}$	FAM	2.0	104-147	23	0.751	0.776	0.053	0.074	0
		R: AATAAAATAGCATTCCATGTGTAGC										
bcLK211	Isoda &	F: CCATTCTCCATAGGTTCATTG	$(CT)_{16}$	FAM	2.5	174-242	28	0.746	0.745	0.037	0.052	0
	Watanabe, 2006	R: ATGCTCCTTACTAAGTCAGATACAC										
Ld30	this study	F: TTGTAGGTGTGTATGAAAGTTCTG	$(AC)_{18}$	VIC	1.68	100-138	17	0.674	0.730	0.100	0.133	2
		R: TGCCACTCTATTTCCTTAATGCC										
bcLK228	Isoda &	F: CCCTAACCCTAGAATCCAATAA	$(AG)_{18}$	VIC	0.6	165-215	15	0.869	0.837	-0.013	0.064	0
	Watanabe, 2006	R: GAGGAAGGCGACAAGTCATT										
Ld50	this study	F: GAAGGCGACTTTACATGCCC	$(CA)_{18}$	PET	2.2	157-205	19	0.729	0.761	0.066	0.069	2
		R: TCCATCTTTATGTCTCTTCCATGC										
bcLK189	Isoda &	F: ACCATACGCATACCCAATAGA	$(AG)_{17}AT(AG)_6$	NED	1.2	142-172	15	0.733	0.789	0.096	0.074	2
	Watanabe, 2006	R: AGTTTTCCTTTCCCACACAAT										
bcLK253	Isoda &	F: AACACCATAGTGCAATGTGC	$(AG)_{17}$	NED	1.1	195-227	17	0.821	0.806	0.006	0.071	0
	Watanabe, 2006	R: TCCTCTTGTTGATGCCACTT										
Multiplex 2												
Ld58	this study	F: AATGGCAAGAGCAGCAATCC	$(AC)_{15}$	FAM	2.1	131-183	25	0.775	0.815	0.077	0.073	0
		R: TCCAGGAATGATTTATCGAGAGC										
Ld45	this study	F: TGTGGGAGGTATAGCTTGGC	$(CA)_{13}$	FAM	2.25	198-216	12	0.653	0.723	0.121	0.092	2
		R: AGTAGGATGGAATGATGGAAACAC										
Ld42	this study	F: TCGTATGCATTGTCCAAATTTCC	$(TG)_{14}$	VIC	1.8	167-191	9	0.551	0.590	0.084	0.134	1
		R: TCCAAGTGAGGTCACACGAG										
bcLK263	Isoda &	F: CGATTGGTATAGTGGTCATTGT	$(TC)_{20}$	PET	3.2	185-259	36	0.801	0.868	0.099	0.056	2
	Watanabe, 2006	R: CCATCATACCTTCTTGAAGAG										
Ld101	this study	F: ACACCAAGGACTCTCTGACTAC	$(AC)_{12}$	NED	1.3	179-215	15	0.382	0.395	0.057	0.080	0
		R: GGTGATTCCAGAAGCAGGTG										
Ld56	this study	F: AGCCATCGTGGTTCTTCTTTG	$(AC)_{16}$	NED	1.6	219-247	14	0.749	0.769	0.049	0.099	0
		R: CTTGTAACTGTGCACCCACC										

[C]: primer concentration in the primer premix [µM], N_A: number of alleles, H_O: observed heterozygosity, H_E: expected heterozygosity, F_{IS}: inbreeding index, F_{ST}: fixation index, *Sequence codes for primers: Ld31: lardec012611, Ld30: lardec001529, Ld50: lardec022835, Ld58: lardec022359, Ld45: lardec024823, Ld42: lardec023929, Ld101: lardec025807, Ld56: lardec023228. †number of Mendelian inconsistencies identified by comparing the genotype of the six half-sib families and their mother

the genotypes of the offspring. As there were too few loci combining sufficient polymorphism and zero mismatches in the progeny test, we retained those loci with no more than two Mendelian discrepancies and sufficient polymorphism. The number of possible multiplex combinations was limited as allele size ranges of the markers were large and as we had to take into account heterogeneities in PCR conditions. We were finally able to design one 6-plex and one 7-plex that we validated over the 413 individuals coming from across the range. Laboratory protocols were successfully tested in another laboratory (INRA Orléans, UR AGPF, Vanina Guérin, personal communication), confirming their robustness.

Genotype scoring and analyses

Clear binning of alleles was confirmed over the whole sample. The marker allelograms, i.e. diagrams showing all detected allele raw sizes ranked in increasing order, are shown in Figures 3 and 4. They indicate that most markers are characterized by a clear succession of dinucleotide repeats over large size ranges (between 28 and 74bp, mean 42bp) and a remarkably high number of alleles (9-36, mean of 20). Off-ladder microvariants, i.e. allele sizes that are in between the sizes expected from the repetition of dinucleotide units, were only observed for one of the 13 loci (Ld45, see Figure 4) and as these intermediate size variants were clearly separated from the neighbouring size classes, they did not cause problems for binning. In fact, such clearly defined off-ladder microvariants can improve the precision of the analyses when they are correctly identified (Guichoux *et al.* 2011b). There was no incoherency across repetitions (positive and random controls). Type A and Type B error rates ranged between 0 and 0.19% (mean of 0.05%) for multiplex 1, and between 0 and 1.3% (mean 0.32%) for multiplex 2. The frequencies of cases that could not be scored were slightly higher. They ranged from 0 to 2.1% (mean of 0.32%) for multiplex 1 (mainly caused by an unspecific product being amplified at locus bcLK211) and from 0 to 0.94% (mean of 0.16%) for multiplex 2 (mainly caused by excessive stuttering of some long alleles at locus bcLK263). All markers were highly polymorphic. Number of allele (N_A) observed (H_O) and expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) and coefficient of differentiation (F_{ST}) are shown in Table 2. F_{IS} values were slightly positive except at locus bcLK228, which is an indication of the presence of null alleles. To better estimate the frequency of null alleles, we relied on the software MICRO-CHECKER v.2.2.0.3 (Van Oosterhout *et al.* 2006). The method searches for loci with a significant homozygote excess evenly distributed across all

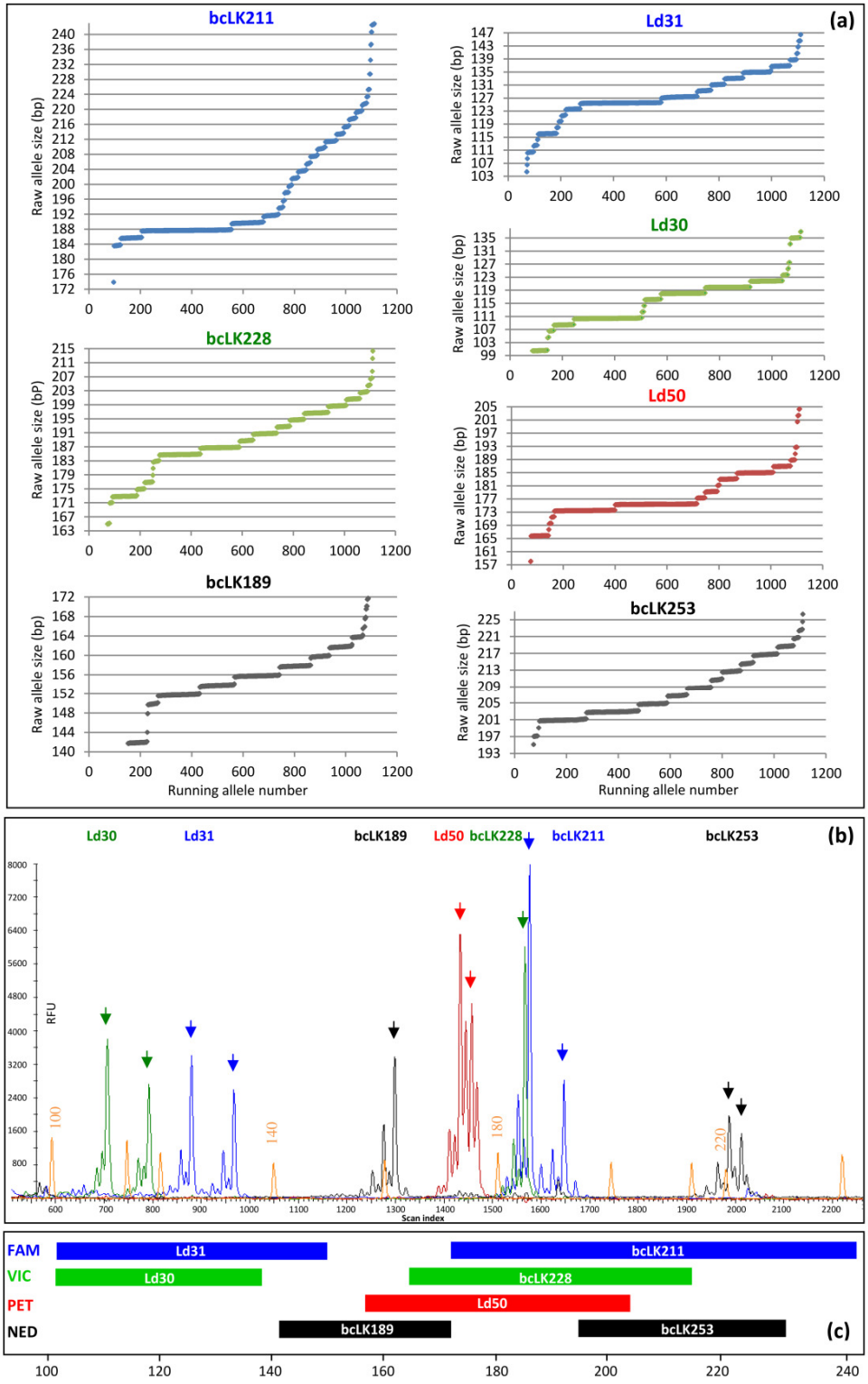

Figure 3 Binning, profiles and marker ranges of SSR multiplex 1. (a) Allelograms based on 413 individuals from across the range. The running allele number corresponds to the position of the allele in the list of allele raw sizes ranked in increasing order. (b) Example of an individual electropherograms. (c) Range sizes based on the same sample as in (a). In (b), arrows point to alleles at each locus, orange peaks correspond to fragments of the internal size standard LIZ600, and numbers above these peaks indicate fragment sizes (base pairs).

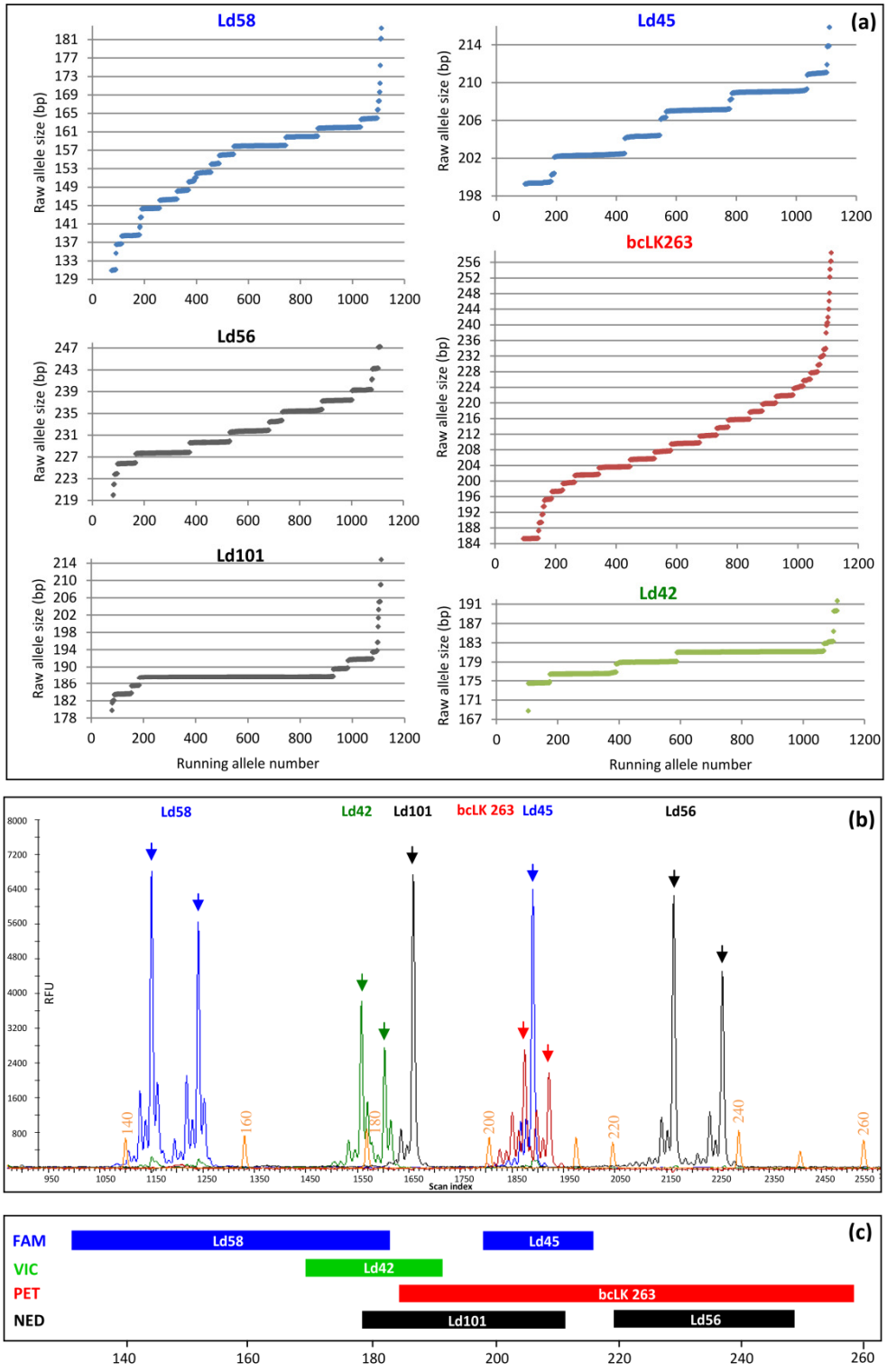

Figure 4 Binning, profiles and marker ranges of SSR multiplex 2. (a) Allelograms based on 413 individuals from across the range. The running allele number corresponds to the position of the allele in the list of allele raw sizes ranked in increasing order. (b) Example of an individual electropherograms. (c) Range sizes based on the same sample as in (a). In (b), arrows point to alleles at each locus, orange peaks correspond to fragments of the internal size standard LIZ600, and numbers above these peaks indicate fragment sizes (base pairs).

homozygote-classes. The analysis of a subsample of 12 populations pointed to 13 cases out of 156 population/marker combinations suggesting the presence of null alleles, involving 7 of the 13 loci (Table S2, Supporting information). The average null allele frequency across all loci per population ranged between 2.4% and 6.6%. As shown in simulation studies of Chapuis & Estoup (2007), null alleles with frequencies between 5-8% should have only reduced effects on classical estimates of population differentiation. Hence, the genotyping results could be used directly with no need of data correction for null allele genotypes. However, as recommended by Oddou-Muratorio *et al.* (2009), correction of null allele genotypes remains of interest for some other analyses. This can be done by using the correction options implemented in different software (e.g. MICRO-CHECKER).

Transferability of the markers was tested in six other *Larix* species and led to heterogeneous results, although new alleles have been identified in all species (Table 4). Note that allelic richness estimates for the different species are not directly comparable as we used different sample sizes. In cases of transfer problems we differentiated four different kinds which are given in the table (a-d) and explained in its footnote. While our datasets did not allow detailed conclusions about success of transferability according to phylogenetic relationships, transfer rate was lower for *L. sibirica.* According to nuclear phylogenies based on AFLP and ITS data (Gernandt & Liston 1999; Semerikov *et al.* 2003), this species forms a clade with *L. decidua* and the other Eurasian species. However, other phylogenies based on chloroplast data separate *L. sibirica* from all other *Larix* species, in agreement with our data (Qian *et al.* 1995; Semerikov *et al.* 2003).

Table 4 Results of transferability in six other *Larix* species. Numbers correspond to allele counts.

Locus	*L. kaempferi** (12)‡	*L. gmelinii** (12)‡	*L. sibirica** (21)‡	*L. laricina*† (10)‡	*L. lyallii*† (4)‡	*L. occidentalis*† (4)‡
Multiplex 1						
Ld31	8	8	5¶	5	2	2
bcLK211	4	4	4	5	2	4
Ld30	–§	–§	–§	–§	3	–§
bcLK228	3	7	9	8	4	-††
Ld50	12	8	3¶	-§	-§	–§
bcLK189	6	8	6¶	-§	2	–§
bcLK253	4	3	3	8	4	3
Multiplex 2						
Ld58	-**	–§	6	5	3	3
Ld45	-**	11	2¶	3	1	4
Ld42	6	7	–§	5	3	6
bcLK263	5	3	15	12	7	6
Ld101	7	12	9	2	2	3
Ld56	9	9	6	2	2	8

*Eurasian species.
†American species.
‡Number of tested individuals in brackets.
§No amplification.
¶Amplification in only a subset of individuals.
**Only one locus (either Ld58 or Ld45) was amplified but it was not clear which one.
†† Profile ambiguities.

Conclusions and perspectives

This study shows that even for species with very large genome size and low overall polymorphism, new highly informative microsatellites can be developed based on 454 sequencing, provided that some precautions are taken. These precautions involve (i) verifying if the initial sequencing of the enriched microsatellite library can guarantee a sufficient read length and sequencing depth to identify enough candidates and (ii) focusing from the beginning on microsatellites with high number of repeats. The two *Larix decidua* multiplexes developed in this study are currently applied on a range-wide sample of 43 populations to resolve in detail past population dynamics, which should help identify and protect valuable genetic resources.

Data Accessibility

DNA sequences: Genbank accessions JQ340312-JQ340319

Sample locations and microsatellite data: DRYAD doi: 10.5061/dryad.08507r35

Supporting Information

Table S 1 Simplex test results for final markers

					Test 1 Polymorphism			Test 2 Null alleles		
Marker	Sequence name	Repeat Type	Nb Repeat Units	Quality of profile	Nb alleles	Nb individuals	Candidate for validation	Test for further validation	Nb alleles	Nb incoherencies
Ld_31	lardec012611	(AC)	18	ok	5	7	Y	progeny test	11	0
Ld_50	lardec022835	(CA)	18	ok	6	8	Y	progeny test	14	2
bcLK211	bcLK211	(CT)	16	ok	10	7	Y	progeny test	17	0
bcLK189	bcLK189	$(AG)_{17}AT(AG)_6$	17+6	ok	7	7	Y	progeny test	13	2
bcLK228	bcLK228	(AG)	18	ok	7	7	Y	progeny test	16	0
Ld_58	lardec022359	(AC)	15	ok	7	7	Y	progeny test	15	0
Ld_56	lardec023228	(AC)	16	ok	8	7	Y	progeny test	12	0
bcLK253	bcLK253	(AG)	17	ok	9	7	Y	progeny test	11	0
bcLK263	bcLK263	(TC)	20	ok	10	7	Y	progeny test	14	2
Ld_42	lardec023929	(TG)	14	ok	5	7	Y	progeny test	8	1
Ld_45	lardec024823	(CA)	13	ok	6	7	Y	progeny test	7	2
Ld_30	lardec001529	(AC)	18	ok	5	7	Y	progeny test	8	2
Ld_101	lardec025807	(AC)	12	ok	4	5	Y	progeny test	7	0

Table S2 MICRO-CHECKER results (see online resources of Molecular Ecology DOI 10.1111/j.1755-0998.2012.03139.x)

CHAPTER 4:

Translocation genetics of European larch

Introduction

Translocations are defined as anthropogenic movements of living organisms from one place to another (IUCN 1987). They increasingly affect the integrity of native plant and animal populations through hybridization and genetic swamping resulting in growing conservation concerns (Allendorf *et al.* 2001; Hufford & Mazer 2003). Despite their importance, they are understudied. In particular, research on translocations is clearly lagging behind that on biological invasions, which has received considerable attention during the last 15 years (for review see Ellstrand & Schierenbeck 2000; Lee 2002; Mooney & Cleland 2001; Sakai *et al.* 2001; Suarez & Tsutsui 2008). Some cases of translocations have been classified as "cryptic invasions" (e.g. Kolbe *et al.* 2004; Kolbe *et al.* 2008; Rosenthal *et al.* 2008; Saltonstall 2002, 2011; Williams *et al.* 2005). However, they represent a rather restricted subset of cases of long-distance, often intercontinental translocations where the introduced material turns out to be selectively favoured. Translocations have broader significance but explicit studies of translocations remain surprisingly limited, except in the particular case of restocking in fishes or of plantations in forest trees (e.g. Deguilloux *et al.* 2003; Gum *et al.* 2006; König *et al.* 2002; Lowe *et al.* 2004; Marie *et al.* 2012; Neville & Dunham 2011; Scribner *et al.* 2000; Winkler *et al.* 2011). To date, none of these relatively few studies has explored in detail translocations across a complete distribution range. Moreover, few have investigated details of the introduction history per se.

To establish a baseline for future research on this topic, it would be useful to identify a biological model with suitable characteristics. First, at least some non-introgressed genotypes should have persisted in most populations, thereby facilitating the identification of introduced or introgressed material. Second, the species should have a strong ancestral genetic structure to facilitate discrimination of native, introduced and admixed genotypes. Finally, a focus on the range-wide scale should help develop a systematic strategy for the detection and sourcing of introduced material, thereby allowing a better understanding of the translocation process.

Trees should be particularly good models for translocations studies as some species have been planted for centuries (Lefèvre 2002). Moreover, many of them are characterized by large populations and long generation time (Petit & Hampe 2006), which should favour persistence of native genotypes. Translocation studies in trees have mostly relied on maternally inherited organelle markers, taking advantage of the strong genetic structure often detected in these species with such markers (Petit *et al.* 2005). However, combining nuclear and organelle data in trees would allow investigating cytonuclear disequilibrium, which can be of even greater

support to detect recent introductions (Asmussen *et al.* 1987). Cytonuclear disequilibrium should be maximal immediately after introduction and then decrease quickly following random mating, as it gets halved at each generation.

A good candidate tree for such a study is European larch (*Larix decidua* Mill.). It mostly grows at high altitudes and is characterized by a restricted native distribution range subdivided into two main areas, the Alpine and the Central European region. This fragmented distribution, whose origin has been investigated using fossils (Huntley & Birks 1983; Lang 1994), should have promoted the development of a strong geographic genetic structure by limiting gene flow across the different parts of the range. Most importantly, the species has been widely planted within and outside its range in Europe since the 16th century, with the intensity of plantations culminating in the 19th century. In Canada and the United States the species has become naturalized at different places (Li & Wyckoff 1994; Little 1979; Seymour 1982; Voss 1972). In Europe, the popularity of European larch as plantation species has reached such a level that it received a name in forest literature: "Lärchenmanie" (German) or "manie du mélèze" (French; Engl. = larch mania) (Fourchy 1952; Münch 1936; Rubner 1953; Schober 1949; Tschermak 1935).

This study focuses on intraspecific translocations using a range-wide *Larix decidua* sample. The issues we address are (i) the systematic detection of translocations using combinations of well-validated methods, (ii) the identification of admixture events in populations where translocations had occured, (iii) the characterization of the frequency and intensity of plantations, (iv) the sourcing of the material used for plantation and finally (v) the reconstruction of the original genetic structure and its comparison with the current (post-plantation) genetic structure.

Material and methods

Plant material and DNA isolation

In view of the long history of plantations involving *Larix decidua* and hence of the dubious status of larch populations in several regions, we carefully selected 40 presumably native populations from across the range (Fig. 5, Table S2, Supporting information), taking advantage of the existence of a multi-site *L. decidua* provenance trial established 1957/1958 (Schober 1977; Schober 1985). This trial includes populations from most parts of the historical range and aims to study important phenotypic traits for forestry (e.g. growth

rate, canker sensitivity). During its establishment special care had been taken to select native populations, taking into account available historical information on plantations (e.g. Münch 1936; Rubner 1953). Moreover, by sampling material gathered 50 years ago, one should reduce the risk of working with non-native populations. Three populations correspond to the variety or subspecies *polonica*, located in the Swietokrzyskie Mountains and listed as vulnerable on the IUCN Red List (Lewandowski 1997). We also included five populations located beyond the acknowledged species distribution range (72, 73, 78, 79, and 80). In 2010 we collected phloem and needle samples from these 45 populations (24 individuals per population). Most of these populations (32, i.e. 71%) originated from eight German provenance trial sites and the remaining ones (13, i.e. 29%) were sampled *in situ*. For comparison, we also included one *L. sibirica* population from a Swedish provenance trial. Details about sampling technique and DNA isolation had been described previously by Wagner *et al.* (2012).

Mitochondrial DNA

To obtain a representative sample for mitochondrial variation, we sequenced eight individuals per population at two mtDNA regions: UBC460 and *atp*A. These mtDNA regions had been successfully used for similar purpose in other *Larix* species (Polezhaeva *et al.* 2010; Semerikov *et al.* 2006). Primer sequences for UBC460 were published in Semerikov *et al.* (2006) while primer sequences for *atp*A (5'-GCGGCTGCCTATAGATACGA-3' and 5'- GCTACCGAGGCAGATATGGA-3') were designed by V. Semerikov (pers. comm.). PCR reactions were performed in a volume of 25 µl with 1× PCR buffer (DreamTaq Green, Fermentas), 0.24 mM of each dNTP, 0.16 mg/ml of BSA, 0.2 µM of each primer, 1 U of Taq polymerase (DreamTaq Green, Fermentas) and 20 ng of genomic DNA. PCR programs started with an initial denaturation at 94 °C for 4 min followed by 30 (UBC460) or 28 (*atp*A) cycles of denaturation at 94°C for 1 min, annealing at 65 °C (UBC460) or 57 °C (*atp*A) for 45 s and elongation at 72°C for 2 min 50 s (UBC460) or 1 min 50 s (*atp*A). The cycles were followed by a final elongation at 72°C for 10 min. PCR products were sent to LGC Genomics for cleanup and sequencing. Sequence editing and alignment was performed using CodonCode Aligner Version 4.0.3. For each mtDNA region, forward and reverse sequences of each sample were aligned and edited, if necessary, according to the strand with the better sequence quality at the respective base position. All edited good quality sequences from each region were aligned using the ClustalW algorithm.

Variable sites were checked for artefacts and sequence errors. A table of confirmed differences was exported and haplotypes were assigned using GENALEX 6.41 (Peakall & Smouse 2006). Using differences from both genes, combined haplotypes were determined. For reconstructing phylogenies and minimum spanning networks, artificial sequences were generated based on the variable sites. The *atp*A region contained a minisatellite with two different repeat motifs and a variable number of repeats. This variation was coded into single nucleotide positions to compute a minimum spanning network using TCS 1.21 (Clement *et al.* 2000) with a fixed 12-step connection limit.

Microsatellite genotyping and cluster analysis

24 individuals per population were genotyped at 13 highly variable microsatellite loci combined in two multiplex following Wagner *et al*. (2012). For subsequent genotype scoring and allele binning based on raw sizes, we used STRAND (http://www.vgl.ucdavis.edu/informatics/strand.php) and AUTOBIN (http://www4.bordeaux-aquitaine.inra.fr/biogeco/layout/set/print/Ressources/Logiciels/Autobin). Bayesian cluster analysis was performed by running STRUCTURE 2.1 (Falush *et al.* 2003; Pritchard *et al.* 2000) with *K* values ranging from one to 10, and 10 runs for each *K*. We worked on the implemented admixture model, a burn-in of 200 000 and subsequent 1 000 000 iterations. To determine the most likely value of *K* we computed the posterior probabilities and the second order rate of change L''(K) with STRUCTURE HARVESTER (Earl & vonHoldt 2012; Evanno *et al.* 2005). We explored the inter-cluster relationships by deriving a neighbour joining tree from the pairwise distance matrix of the STRUCTURE output using the *R* package APE (Paradis *et al.* 2004).

Evaluation of assignment accuracy

To evaluate assignment accuracy we simulated 5000 individuals under different scenarios (cf. Results section) using HYBRIDLAB version 1.0 (Nielsen *et al.* 2006). Subsequently 1000 individuals were chosen at random from these simulations and analyzed with STUCTURE with the same parameters as in the original analysis. We included the original genotypes with assignment values (*q*-values) ≥0.75 as a reference (USEPOPINFO model), so that the assignment scores of the simulated individuals could be compared with those from the observed genotypes in the original analysis. We also included as controls the 263 individuals that had *q*-values <0.75, to check if assignment scores remain the same across analyses.

Cytonuclear disequilibrium and nuclear admixture analysis

In the combined mito-nuclear analysis we tested for the association between mitochondrial and nuclear lineages. We used a *z*-test to compare admixture proportions among mitochondrial groups and a one-sided student test to compare *q*-values among populations with and without indication of recent translocations (cf. Results section).

Translocation frequency, intensity and source areas

We compared observed translocation frequencies with theoretical expectations from the Poisson law and tested if there was a difference with a χ^2-test. Existence of regional differences in translocation frequency was tested by comparing proportions of purebred and admixed individuals found in the Alpine and the Central European region using a *z*-test. To test if translocations often involved multiple sources, we counted the number of different nuclear cluster per population and compared results among populations with and without indication for translocation using a Wilcoxon rank-sum test.

Systematic detection of first-generation migrants

To detect cases of recent translocations separately in each population, we applied a Bayesian approach implemented in GENECLASS (Piry *et al.* 2004). It allows identifying first-generation migrants in a population and provides likelihoods of belonging to a given reference population. With this approach, recent translocations, especially those across very divergent populations, are assimilated to first generation migrants. If admixture has taken place, the model assumptions are not longer fulfilled and introduced genotypes should no longer be identified. We relied on 10,000 simulated individuals (Paetkau *et al.* 2004) and a type I error of 0.01. We ran GENECLASS three times with the same parameters. After each run, we excluded the detected migrant individuals.

Composition of introduced material and reconstruction of ancient genetic structure

To investigate the composition of introduced material, we calculated mean *q*-values of each cluster for different groups of introduced individuals corresponding to different combinations of source and release areas. These were compared to mean *q*-values of presumed native Alpine and Central European populations that were obtained by removing individuals

considered to result from translocation or associated admixture (identified using either STRUCTURE or GENECLASS, cf. Results section). The remaining individuals were mapped and summary statistics including classical measures such as allelic richness (N_A), gene diversity (H_S), inbreeding coefficient (F_{IS}) and fixation index (F_{ST}) were calculated and compared with the original using FSTAT version 2.9.3.2 (Goudet 2001) with 15,000 permutations for nuclear data and using PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/) for mtDNA data (Pons & Petit 1996).

Results

Mitochondrial DNA

A total of 22 mtDNA haplotypes were detected in 363 *Larix decidua* samples from 43 populations, 10 of which resulted from simple sequence variation and 12 from minisatellite variation (Fig. 5A). The eight *L. sibirica* samples had a single private haplotype (haplotype 23) that was however not very divergent from those found in *L. decidua*. The *L. decidua* haplotypes formed two main groups, each of which was composed of two frequent haplotypes and of some less frequent ones. In group 1 (haplotypes 16, 17, 18 and 22), haplotypes 16 and 18 were found in 58% of the individuals. In group 2 (haplotypes 8, 9, 10, 19, 20, 21 and minisatellite variants), haplotypes 9 and 10 occurred in 28% of the individuals. Haplotypes of group 1 were mostly found in the Alpine region and those of group 2 in Central Europe (Fig. 5B). However, there were 37 individuals (10%) with group 1 haplotypes found in Central Europe and 14 individuals (4%) with group 2 haplotypes found in the Alpine region. These individuals were scattered across the range and typically interspersed in populations of the regionally common haplotypes. Two populations were composed solely of regionally uncommon haplotypes: populations 73 and 79, in Central Europe. Both were located outside the acknowledged specie range. The strong geographic structure of mtDNA separating the Alpine and the Central European region and the sporadic occurrences of haplotypes not fitting with this pattern raise the question of whether this is caused by ancient gene flow or by recent translocations. To study this question, we analysed high resolution nuclear data and compared them with the mtDNA data.

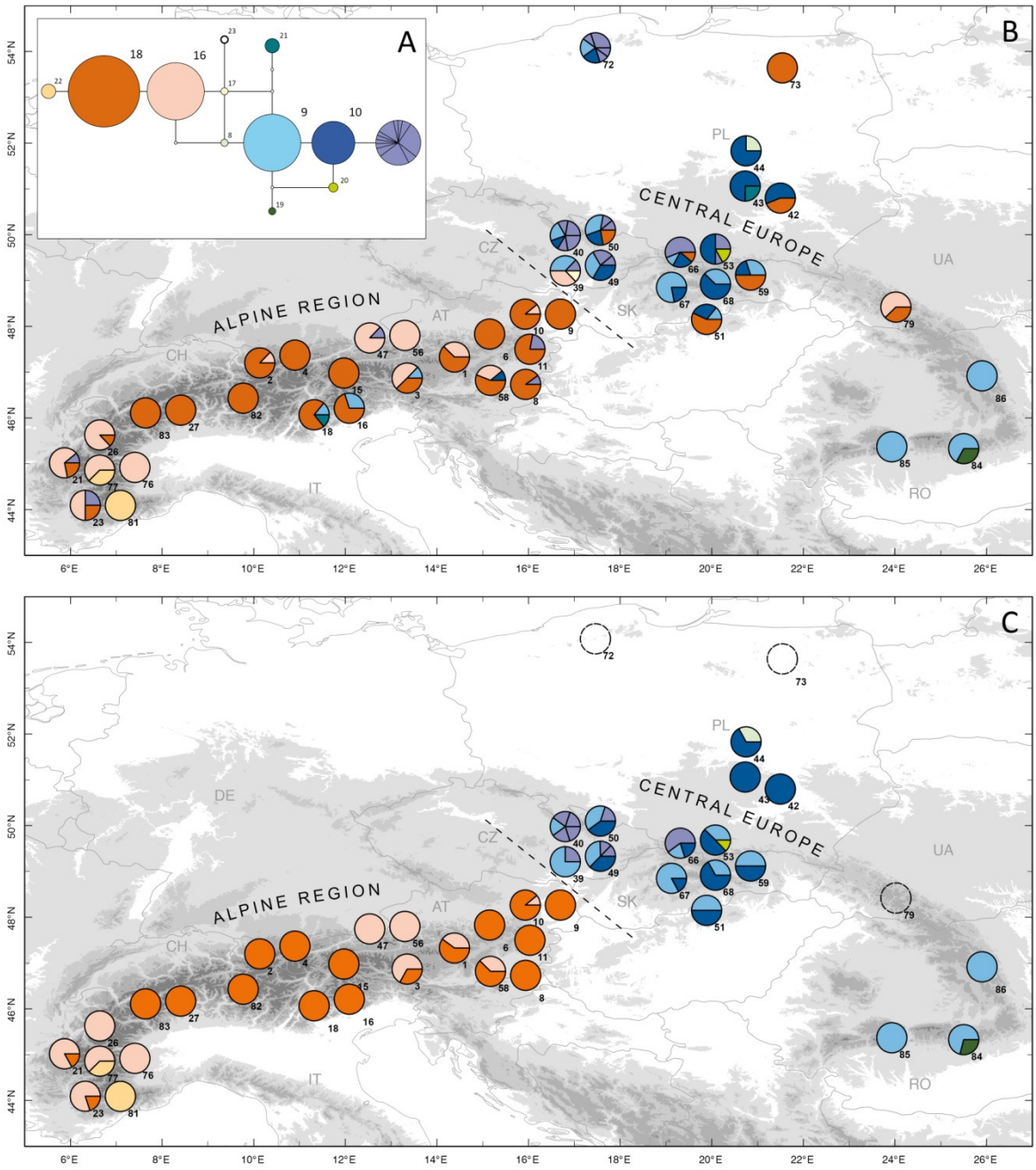

Figure 5 Minimum spanning network (A) and distribution of the combined mitochondrial haplotypes (atpA and UBC460) before (B) and after (C) systematic translocation removal. In (A) circles represent haplotypes labelled by their codes and scaled to their frequencies. The purple pie chart summarizes haplotypes caused by minisatellite variation. Unlabelled circles symbolize predicted haplotypes that were not observed. Branches correspond to single mutations regardless of their length. In (B) and (C) circles represent the haplotype composition of populations (~8 individuals/population). Labels are population codes.

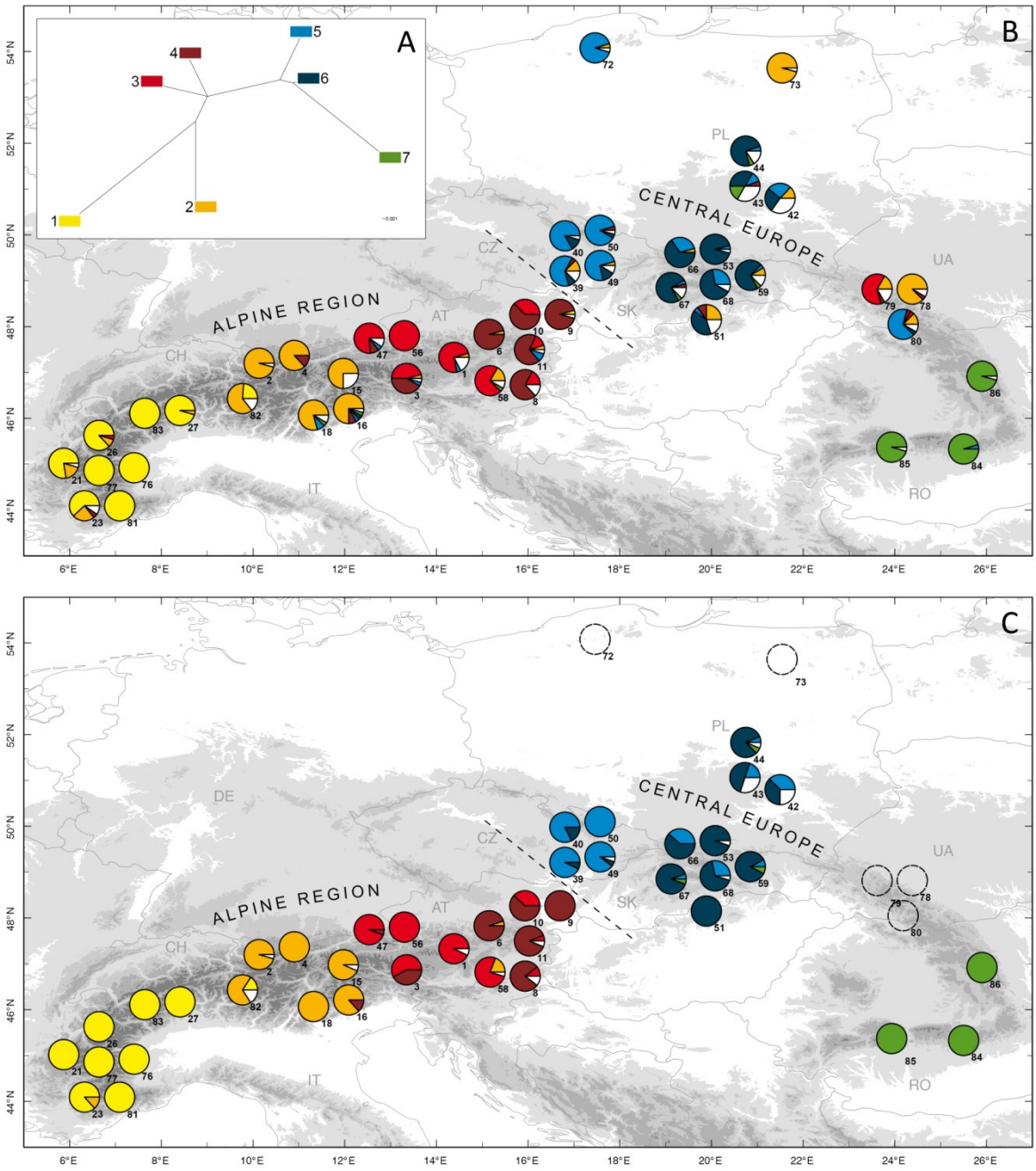

Figure 6 (A) Neighbour joining tree and distribution of the seven SSR clusters detected by STRUCTURE (B) before and (C) after systematic translocation removal. In (A) each rectangle represents a cluster. In (B) and (C) pie charts represent cluster composition of the populations (~24 individuals/population). Individuals with q values >0.5 are coloured, the remaining ones are white.

Nuclear DNA

Cluster analyses of 1026 *L. decidua* individuals from 45 populations using STRUCTURE and calculation of second order rate of change gave strongest support for the existence of three ancestral groups ($K = 3$, Fig. S1, Table S3, Supporting information). However, some statistical support and clear individual assignments were also found for $K = 7$ (Fig. 6A, Fig. S2, Supporting information). The seven clusters were mainly distributed mainly in an east-west direction (Fig. 6B). Yet, in some populations, there were individuals that had high assignment probability to clusters more frequent in other regions. For instance, in population 18, there were three individuals assigned to clusters 5 and 7, whereas all other individuals where assigned to cluster 2. Furthermore, three Central European populations (73, 78 and 79) were entirely composed of individuals assigned to clusters 2 and 3, which were more frequent in the Alpine region. For subsequent analyses, we combined the seven nuclear clusters into two larger groups of related clusters (group 1: cluster 1-4, group 2: cluster 5-7; Fig. S2, Supporting information).

Evaluation of assignment accuracy

To evaluate the robustness of the assignments, we simulated genotypes using HYBRIDLAB, considering two scenarios. One was based on panmixia within each of the seven identified cluster. In the other scenario, we simulated panmixia within the two cluster groups. To derive allelic frequencies for each cluster or cluster group, genotypes from the original sample were selected according to their *q*-values in the STRUCTURE analysis. We selected individuals with *q*-values of ≥ 0.75 for the respective cluster and ≥ 0.875 for the respective cluster group (Alps versus Central Europe), the latter being the sum of the *q*-values of all individual clusters making up the respective cluster group. A threshold value of 0.875 corresponds to the optimal theoretical assignment threshold to distinguish backcross from purebred individuals, whereas the relaxed threshold of ≥ 0.75 used for each of the seven individual clusters corresponds to the optimal threshold to distinguish F1 from purebreds. In the second scenario, individuals were selected only based on *q*-values ≥ 0.875 for cluster groups, i.e. it included all individuals admixed among clusters belonging to the same cluster group. Simulations were used to quantify the frequency with which genotypes produced under each scenario were falsely assigned to the alternative purebred category ($q \geq 0.875$) or to the admixed category ($0.125 \leq q < 0.875$) (Table S4, Supporting information).

We found that, under both scenarios, not a single individual was assigned to the alternative purebred category, pointing to a very low risk to misassign purebred category. In contrast, some purebred individuals were falsely assigned to the admixed category. Their proportion depended on the cluster and on the scenario but it remained limited. Genotypes from group 1 were falsely assigned to the admixed category in 1.3-4.6% of the cases in the first scenario and in 13.3% in the second scenario. For group 2, values ranged between 5.4% and 11.1% in the first scenario and reached 12.3% in the second. Comparison of the 263 original admixed genotypes included in the STRUCTURE analysis and in the subsequent run with the simulated genotypes revealed that there was no important change in the *q*-values between the two analyses (mean difference of 0.034), indicating that the addition of simulated genotypes did not modify clustering criteria, as expected given our settings in the STRUCTURE analysis (USEPOPINFO model).

Cytonuclear analysis

We checked for association between the two nuclear cluster groups and the two mtDNA lineages using 363 individuals for which both type of information was available. All nuclear genotypes assigned to group 1 purebreds carried group 1 mtDNA haplotypes, and all but one group 2 purebred carried group 2 mtDNA haplotypes, indicating nearly total cytonuclear association (Table 5, bold numbers first two lines). We also analysed the same association within each region. In both cases, regionally uncommon nuclear purebreds were associated with regionally uncommon mtDNA lineages (Table 5, bold numbers lines 4 and 5), which we interpret as evidence for recent establishment of genotypes originating from the other region. We then focused on admixed individuals in both regions. We reasoned that if introgression was caused by translocations, there should be a higher proportion of introgressed individuals characterized by the regionally uncommon mtDNA lineage (i.e. presumably introduced) than by the regionally dominant lineage. In the Alpine region we found 14 individuals with group 2 haplotypes. Seven of them (50%; Table 5, underlined) were introgressed ($0.125 \leq q < 0.875$). This contrasts with the corresponding proportion in individuals with group 1 haplotypes (13%, z = -3.68, $p < 10^{-3}$). In Central Europe, results were similar. A total of 37 individuals had group 1 haplotypes and 16 of them (43%, Table 5, underlined) were introgressed, whereas the proportion of introgressed individuals with group 2 haplotypes was significantly lower (24%, z = -2.26, $p = 0.03$). Altogether, mtDNA and nuclear data cross-supported each other, suggesting that in both regions, uncommon nuclear and mitochondrial

Table 5 Counts of mito-nuclear genotype combinations for different spatial scales (gr = group)

		nuclear gr 1 purebred	nuclear gr 2 purebred	nuclear gr admixed	nuclear gr total
Alps + Central Europe	**mt gr 1**	**180**	1	40	220
	mt gr 2	0	**104**	38	143
Alps	**mt gr 1**	160	0	24	184
	mt gr 2	0	**7**	7	14
Central Europe	**mt gr 1**	**20**	1	16	37
	mt gr 2	0	97	31	128

lineages resulted from translocations. We decided to use only the nuclear criteria for the detection of translocations and of admixture events, as nuclear data was available indistinctly for all genotypes, in contrast to mtDNA. Using the nuclear criterion for purebreds, we detected 89 (8.6%) exotic genotypes (i.e. purebreds belonging to the cluster group that is regionally uncommon), of which 23 were found interspersed in populations with common genotypes and 66 in the three populations predominantly composed of exotic genotypes (Fig. 7, black coloured proportions).

Nuclear admixture analysis

We reasoned that in populations where translocation events had been detected, more admixed individuals should be found than in populations where no translocation event was detected. To test this hypothesis, populations were assigned into two categories: one with at least one exotic genotype, the other one with populations lacking such evidence. The analysis was also performed separately in each region (Alps, Central Europe). In each population studied we excluded purebreds originating from the other region to direct the analysis on admixture. The prediction was that in populations with indication of translocation, average *q*-values should be higher. In both regions differences in levels of admixture between the two categories (with versus without evidence for translocation) were highly significant (Alps: *q*-value = 0.08 versus 0.05, $p<10^{-4}$, Central Europe: *q*-values = 0.19 versus 0.09, $p<10^{-4}$, Fig. 8). This suggests a relationship between translocation and admixture. Overall we found 155 (15%) admixed individuals using the selected thresholds (Fig. 7, grey coloured proportions).

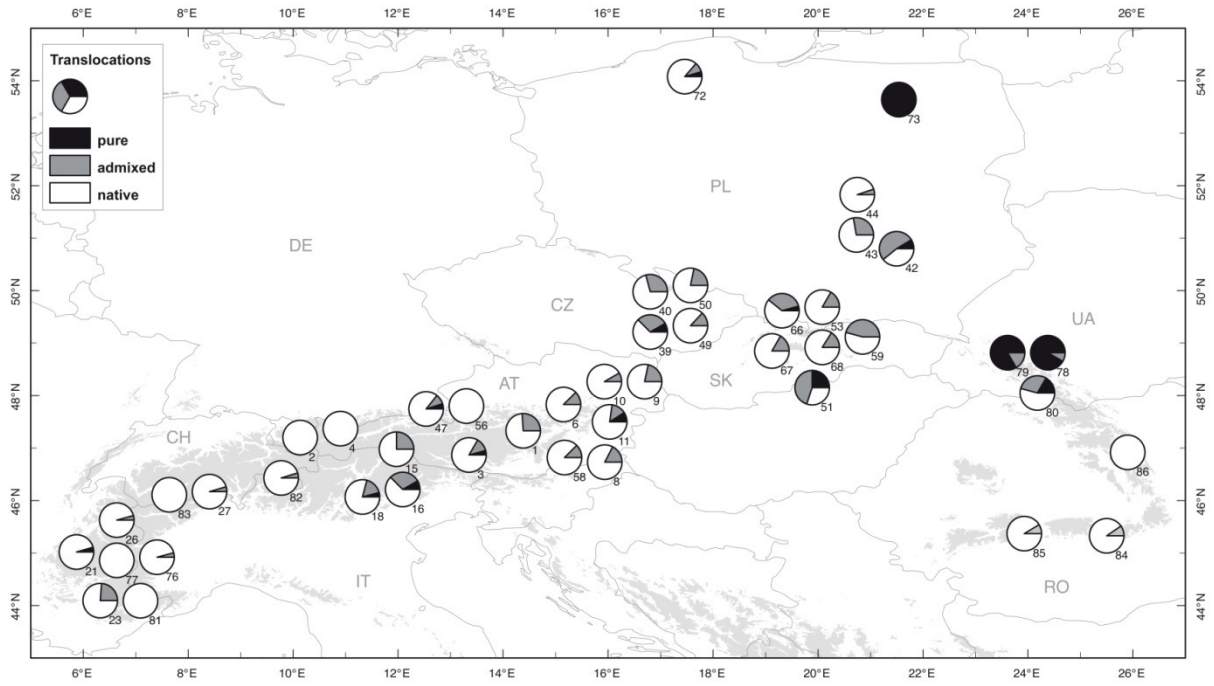

Figure 7 Distribution of opposite group purebred and regionally admixed individuals based on STRUCTURE assignments

Translocation frequency, intensity and number of source areas

We then tried to better describe the process of translocation, i.e. its frequency, intensity and distribution. First, we tested if the probability of occurrence of translocation events can be considered to have the same probability across populations or if it is higher in some populations than in others. For this purpose, we counted the number of translocation events per population, excluding the five non-native populations. The number of introduced individuals per population ranged from zero to five (mean = 0.5). As the overall frequency of exotic individuals was low (2%), we considered translocations as rare events and compared their distribution with the theoretical expectations derived by the Poisson law. The distribution did not match with Poisson law predictions ($\chi^2 = 259$, $p \approx 0$; Fig. S 3, Supporting information). Observed frequency of populations with individual introduced was lower than expected whereas the observed frequency of populations with 0, 2 and 5 introduced individuals was higher than expected. This points to a deficit of cases with no or few introductions and an excess of cases with multiple introductions, compared to a scenario where introductions would have taken place with the same probability across all populations. Second, we checked if translocations were equally abundant in the two regions (Alps, Central Europe). In each region, we compared the proportion of exotic purebreds and of regionally admixed individuals. For both values the proportion was significantly lower in the Alps than

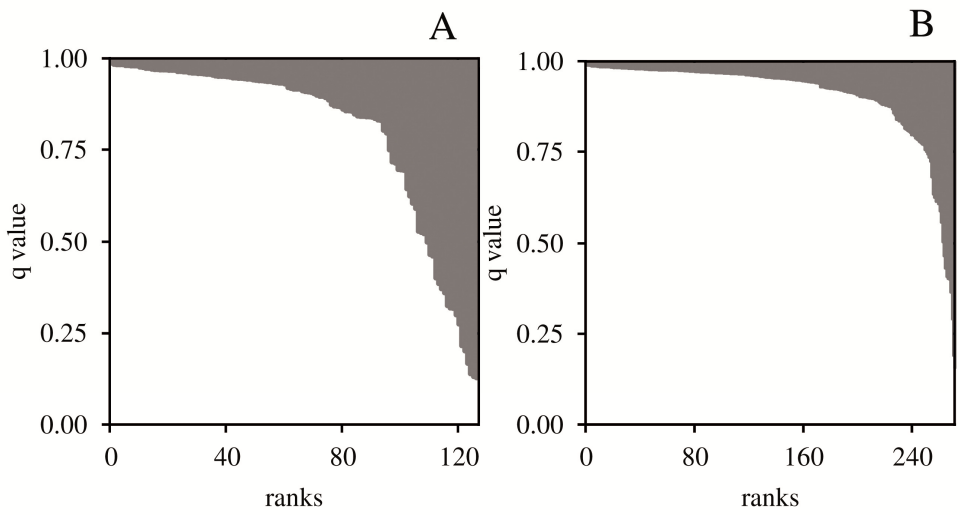

Figure 8 Admixture in populations (A) with and (B) without indication for recent translocation in Central Europe. Bars represent individual q-values, in grey, group 1, in white group 2.

in Central Europe (2% versus 5%, z = -3.04, p°=°0.002 and 10% versus 37%, z = -8.43, p°<10^{-5}, respectively). Third, we checked if translocation events involved material from a single source area or from several ones. For this purpose, we focused on the Alpine region where geographic genetic structure is stronger than in Central Europe. As previously, we compared populations with and without indication of translocation events. In each population, the clusters were counted on the basis of a critical q-value of ≥ 0.5. In the group where no exotic individual was detected, we considered all clusters minus one (the most frequent one). In the group where exotic individuals were detected, we considered all clusters minus two (the most frequent in the population and the most frequent among introduced individuals, i.e. assuming introduction from a single geographic origin). We then compared the number of extra clusters found in the two groups. We found a mean number of 0.8 and 1.8 extra clusters in populations without and with translocation events, respectively. To test if cases of interregional translocations were associated with cases of intraregional translocations, we performed the same comparison but focusing only on extra Alpine clusters. We found in this case on average 0.7 and 1.2 extra clusters in populations without and with translocation events. Differences were significant in both cases (p<0.001), suggesting that introductions often involve material from several geographic sources or from already admixed sources.

Systematic detection of first-generation migrants

The approach based on STRUCTURE could only be applied at the interregional scale due to a lack of suitable mtDNA variation at finer scales to validate it. However, there is no reason to believe that translocations have not taken place also within each region, as suggested by the study of the number of extra clusters in populations with indication of translocation. To recover other types of translocation events, we used the GENECLASS approach (Piry *et al.* 2004). Three successive runs led to the detection of 92 (9%) first-generation migrants among all samples (Fig. 9). A total of 40 of these 92 individuals corresponded to interregional translocation events, only 13 of which had been previously detected. The remaining 52 individuals corresponded to intraregional translocation events. The classification of translocation events was based on the likelihoods of each multilocus genotype to belong to each cluster group or each of the seven clusters. The intraregional translocations could be further subdivided in 23 inter- and 28 intra-cluster translocation events. The latter occurred mostly (8 out of 12 cases) together with translocation events at larger geographic scales. We checked if the detection power of the GENECLASS approach was lower in populations in which admixture had been detected with the previous approach. We found that this was the case: in strongly admixed populations, translocations typically remained undetected (Fig. S 4) which can also explain why we detected only one third of the translocations detected previously, as mentioned above.

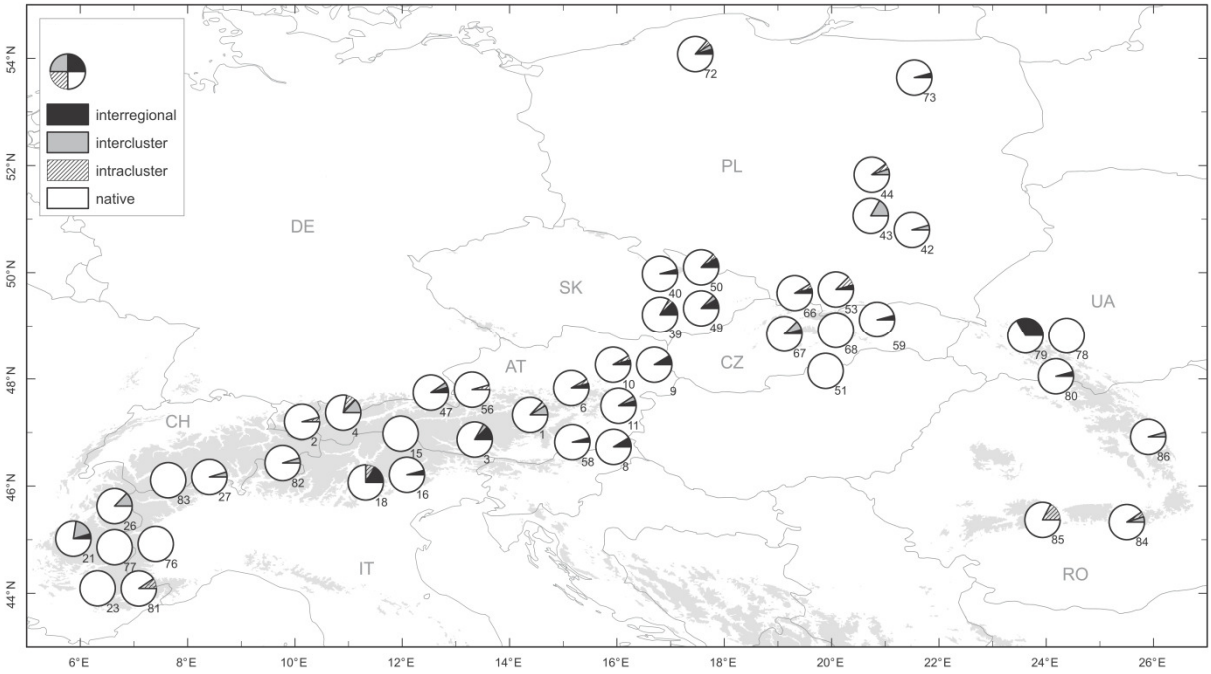

Fig. 9 Distribution of first-generation migrants detected by GENECLASS. Spatial classification based on the likelihood calculations.

Composition of introduced material and reconstruction of ancient genetic structure

To investigate the genetic composition of introduced material, we assigned all exotic genotypes into four categories representing the four possible combinations of source and release areas (Alps to Central Europe, Central Europe to Alps, intra-Alps, intra-Central Europe). For consistency, we considered only individuals with q-values ≥0.875 for one of the two cluster groups. The comparison between presumably natural populations of the Alpine region and material inferred to have been introduced from the Alps revealed that genotypes from cluster 2 were more frequently involved than genotypes from other Alpine clusters (intra-Alps: 48%, Alps to Central: 60%; Fig. S 5, Supporting information). For material introduced from Central Europe, genotypes from cluster 5 were frequently involved, especially considering the limited distribution of native populations where this cluster predominates (intra-Central Europe: 24%, Central Europe to Alps: 39%).

Altogether, using both approaches (STRUCTURE and GENECLASS), 322 (31%) individuals distributed in 43 populations were removed in an attempt to recover the original genetic structure (Fig. 5 C, Fig. 6 C). This included exotic purebreds and admixed individuals detected by the STRUCTURE approach as well as the individuals considered to be first generation migrants using the GENECLASS approach. Four of the five populations sampled beyond the acknowledged species range (populations 73, 78, 79 and 80) did not match with the overall pattern. The fifth populations (72) had high heterozygote deficit (F_{IS}=0.09), and no distinct genetic composition despite its location away from the main species range. Thus, we excluded these five populations. Global summary statistics for nuclear data with the original sample ($N_A = 6.6$, $H_S = 0.76$, $F_{IS} = 0.05$, $F_{ST} = 0.08$) and with the final sample ($N_A = 6.2$, $H_S = 0.74$, $F_{IS} = 0.02$, $F_{ST} = 0.11$) differed significantly ($p < 0.001$ for all measures), confirming the need to systematically remove anthropogenic artefacts to correctly estimate genetic parameters. After correction, a few populations still have exceptionally high heterozygote deficit ($F_{IS} > 0.08$: populations 3, 53, 66 and 84, see Table S 6, Supporting information). For at least some of them, the existence of so far cryptic intraregional translocations appears likely on the basis of mtDNA or nuclear variation, pointing to the need for further refinements of the approach. Similarly, for mtDNA, genetic diversity was lower and genetic structure higher with the final sample, obtained after removing introduced material ($h_S = 0.25$, $G_{ST} = 0.67$) than with the original one ($h_S = 0.37$, $G_{ST} = 0.52$).

Discussion

Systematic detection of translocations

Selecting appropriate methods and tools to detect translocations and investigate their consequences remains challenging as this implies differentiating conspecific individuals originating from different parts of the range. Clearly, in most cases individuals from nearby populations should be more difficult to differentiate than those originating from distant locations. In our study, we took advantage of suitable features of European larch, including strong ancestral genetic structure at both mitochondrial and nuclear genomes and the persistance of native genotypes in most populations. We detected two mitochondrial lineages differentiating most individuals from the Alpine and the Central European parts of the range. Scattered occurences of mtDNA haplotypes that did not fit with this pattern suggested that translocations might had been involved. In accordance with this, individuals with mtDNA haplotypes not belonging to the regionally frequent lineage also happened to be characterized by genotypes assigned to nuclear clusters more frequent in the other region. This is a very strong evidence in favour of recent contact and hence of the artificial nature of the mixed populations as a few generations should erase such cytonuclear disequilibrium. The methodology for the detection of cases of long-distance translocation, based on 13 highly polymorphic microsatellites and a comprehensive screening across the range, provides clear-cut results, with virtually no risk to misassign purebred genotypes from one region to the other region. However, such a strategy only allowed differentiating individuals originating from the Alps versus from Central Europe, i.e. at the interregional scale. Yet we showed that, in the Alpine range, translocations from Central Europe were associated with the introduction of material from other parts of the Alpine region. This suggests that translocations are often complex, with material introduced ultimately coming from several sources, both distant and less distant ones.

To systematically detect recent translocations across all scales, we used an approach based on assignment criteria to exclude reference populations as the origin of diploid individuals on the basis of multilocus genotype data (Piry *et al.* 2004). With this method, we detected 92 cases of recent translocations across the range, from the most distant to the most local. The advantage of this approach is that it can be used for species lacking a strong geographic genetic structure as it works on the basis of data from the population of interest only. Although in principle first-generation migrants can also occur naturally, the broad geographic sampling scale of the study makes this hypothesis unlikely in our survey. However, in

situations where native and introduced genotypes have resulted in admixture, the approach failed to detect introduced material, indicating that both methods used have limitations and that future developments to detect non-native genotypes should focus both on comparisons with other populations and on analyses of the internal structure of each target population.

Admixture events

Numerous admixture events were detected, representing 15% of the individuals. Although some false positives are certainly included, admixture events were overall more frequent in populations where translocations were detected. In some populations, introduced and admixed individuals made up more than 50% of the individuals, indicating massive plantation or high success of introduced genotypes in subsequent generations. This finding points to relatively ancient translocation events, in line with the known history of plantations of the species in Europe (e.g. Fourchy 1952; Münch 1936; Rubner 1953; Schober 1949; Tschermak 1935).

The translocation process

The admixture corresponds to the later steps of a translocation. Although it is conditioned by the initial input of exotic material, it is a largely autonomous process. In this respect, it can be compared with the expansion phase of a biological invasion. In contrast, the translocation *per se* depends on human agency and can be compared with the first establishment step of an invasion. We found that plantation pressure had been very heterogeneous across the range, with some areas where human input has been reduced and others where plantations have been frequent. In fact, exotic material was introduced at particularly high rates in Central Europe. Compared to the Alpine region, the native distribution of European larch in Central Europe is particularly fragmented with populations of rather small size, making them more vulnerable to genetic swamping/admixture.

Translocations and associated processes uncovered in this study can be assumed to be related to the intensive periods of larch plantation during the 18th and 19th century described in forest literature (Fourchy 1952; Münch 1936; Rubner 1953; Schober 1949; Tschermak 1935). The high proportion of admixed compared to purely exotic genotypes and their widespread distribution suggest indeed that at least one generation has passed following the first introductions. Moreover, translocations typically involved multiple geographic sources with some genetic clusters being overrepresented, especially clusters 2 and 5. This result is

supported by historical information indicating that larch seeds from Tyrol (a region located in the Italian and Austrian parts of the Alps where cluster 2 is dominant) and the Sudetes (where cluster 5 is dominant) have preferentially been used in plantations (Münch 1936). Hence, our results shed new light on the "Lärchenmanie" by uncovering its irreversible long-term consequences through a modification of the genetic structure of the species, including areas where threatened gene pools have been recognized (e.g. "variety *polonica*" listed on the IUCN Red List).

Reconstruction of ancient genetic structure

Our sampling included five populations sampled away from the acknowledged species range, but whose exotic status is sometimes questioned. No argument was found to modify the currently acknowledged species distribution range in Europe. The genetic structure at nuclear and especially mtDNA that was revealed after removing the individuals considered to have been introduced or to be the result of admixture was much stronger than that found prior to removing these individuals. Lower within population diversity and greater F_{ST} and overall geographic structure were noted. The pre-plantation genetic structure is likely even stronger as our method did not allow us to detect all cases of intraregional translocations or admixture events and as there is still a relatively high heterozygote deficit in some populations, pointing to Wahlund effect due to subpopulation structure possibly caused by plantation

Conclusions and Perspectives

Our study revealed several aspects of the translocation process in the European larch. Although it is to our knowledge one of the most comprehensive studies of translocation and of its consequences, it did not cover all possible aspects. Among the issues that still remain to be addressed, one can cite the development of specific demo-genetic methods to identify simultaneously recent introductions and admixture and their timing. For instance, determination of the earliest translocations and of the peak period of plantations would help determine if they match with historical records. Another important objective would be to test if introduced genotypes or genes have differential fitness compared to local ones. Our results underpin the importance of investigating translocations from a range-wide perspective as this is the only way to efficiently and accurately trace introduced material of different geographic origins.

Supporting Information

Table S 2 Sampling information of the 45 studied *Larix decidua* populations

ID	Lat dd	Lon dd	Alt	Provenance	Country	Trial location	Nb SSR	Nb mt
1	47.33	14.53	1350	Moederbrugg	AT	Münden	23	8
2	47.20	10.67	1100	Schoenwies	AT	Münden	24	8
3	46.87	13.35	900	Muehldorf	AT	Riedesel	24	8
4	47.37	10.90	1150	Fernpass	AT	Münden	23	8
6	47.83	15.15	800	Langau 45	AT	Münden	24	7
8	47.63	15.77	1200	Semmering	AT	Riedesel	23	10
9	48.05	15.93	610	Lammerau	AT	Münden	23	9
10	48.12	15.93	560	Neulengbach	AT	Winnefeld	24	9
11	47.50	16.03	1000	Wechselgebiet	AT	Münden	22	9
15	46.98	11.97	1100	Bruneck	IT	Münden	20	10
16	46.32	11.45	1200	Cavalese	IT	Riedesel	24	10
18	46.07	11.32	600	Tenna	IT	Münden, Riedesel	24	7
21	45.02	6.93	1900	Pragelato	IT	Riedesel	22	9
23	44.78	6.90	1560	Embrun, Aiguilles	FR	Riedesel	21	8
26	44.87	6.65	1400	Briancon, de Villard	FR	Riedesel	23	8
27	46.17	7.83	1550	St Niklaus	CH	Riedesel	22	8
39	49.83	16.97	400	Zabreh-Dubicko	CZ	Riedesel	24	8
40	49.98	16.90	480	Ruda nad Moravou	CZ	Riedesel	24	9
42	50.80	21.10	347	Gora Chelmova	PL	Sellhorn	23	9
43	51.07	20.73	330	Blizyn	PL	Sellhorn	18	8
44	51.83	20.75	180	Mala Wies	PL	Riedesel	19	8
47	47.47	13.10	830	Bluehnbachtal	AT	Spießingsho	21	8
49	50.05	17.55	550	Krnov	CZ	Riedesel	23	9
50	50.10	17.57	450	Krnov	CZ	Münden	23	9
51	49.00	19.88	800	Cierny Vah	CZ	Münden	20	7
53	49.13	20.18	1200	Smokovec	CZ	Riedesel, Spießingshol	24	12
56	47.80	13.30	700	Fuschlsee	AT	Oerrel	23	8
58	46.82	15.17	800	Deutschlandsberg	AT	Münden	24	9
59	49.12	20.82	830	Brezovicka	CZ	Münden	24	10
66	49.00	19.88	750	Cierny	CZ	Münden	23	9
67	48.85	19.12	850	Stare Hory	CZ	Münden, Oerrel	24	9
68	48.92	20.08	1400	Liptovska	CZ	Rotenburg	25	8
72	54.08	17.46	184	Rekovo	PL	*in situ*	22	10
73	53.64	21.54	132	Ruciane-Nida	PL	*in situ*	24	9
76	44.92	6.72	1500	Briançon, Montgenèvre	FR	*in situ*	24	8
77	44.86	6.64	1500	Briançon, de Villard	FR	*in situ*	25	8
78	48.65	24.38	423	Solotwyn	UK	*in situ*	24	-
79	48.42	24.02	1100	Brusturjany	UK	*in situ*	24	8
80	48.05	24.18	500	Rahiv	UK	*in situ*	24	-
81	44.09	7.09	1070	Vallée de la Tinée	FR	*in situ*	21	7
82	46.43	9.77	2000	Sils Maria	CH	*in situ*	21	8
83	46.11	7.64	1800	Zinal	CH	*in situ*	21	6
84	45.33	25.50	1500	Sinaia Forest District	RO	*in situ*	21	9
85	45.37	23.93	1000	Voineasa Forest District	RO	*in situ*	23	8
86	46.92	25.90	1500	Bicaz	RO	*in situ*	24	6

Table S 3 Δk statistics computed with STRUCTURE HARVESTER (Earl & Holdt 2012)

K	LnP(D)	L(K) sd	delta K
2	-57237.39	2.13	369.26
3	-55646.24	2.17	530.14
4	-55204.74	1335.02	0.19
5	-55010.99	2805.84	0.35
6	-53846.35	186.71	4.16
7	-53458.51	18.51	9.28
8	-53242.43	117.09	0.63
9	-53100.08	97.94	0.45

Figure S 1 Δk statistics computed with STRUCTURE HARVESTER (Earl & Holdt 2012)

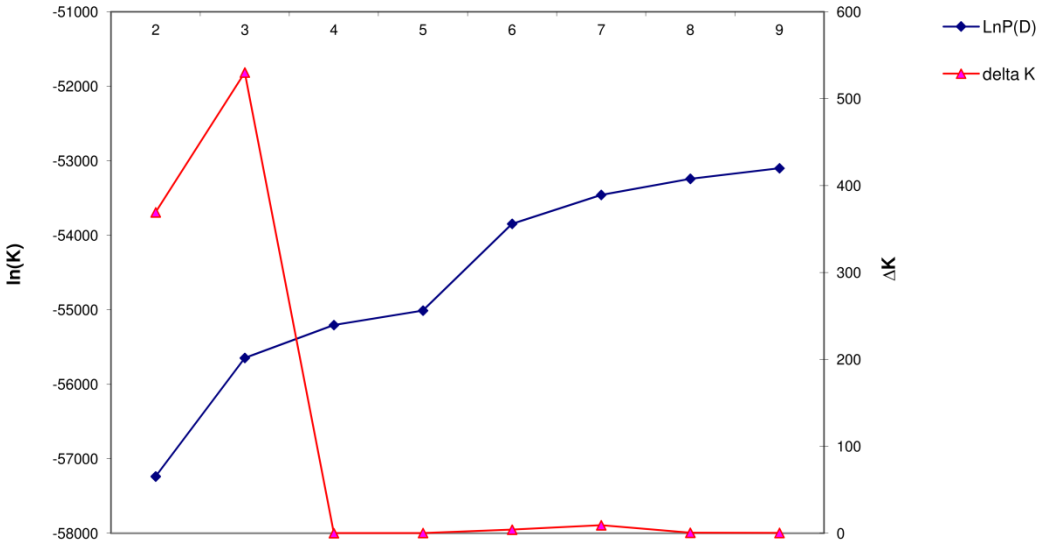

Figure S 2 Individual bar plots based on the STRUCTURE assignments. (A) All seven nuclear clusters and (B) two nuclear groups. Individuals are grouped by population in west-east direction. Each bar represents an individual in its cluster composition.

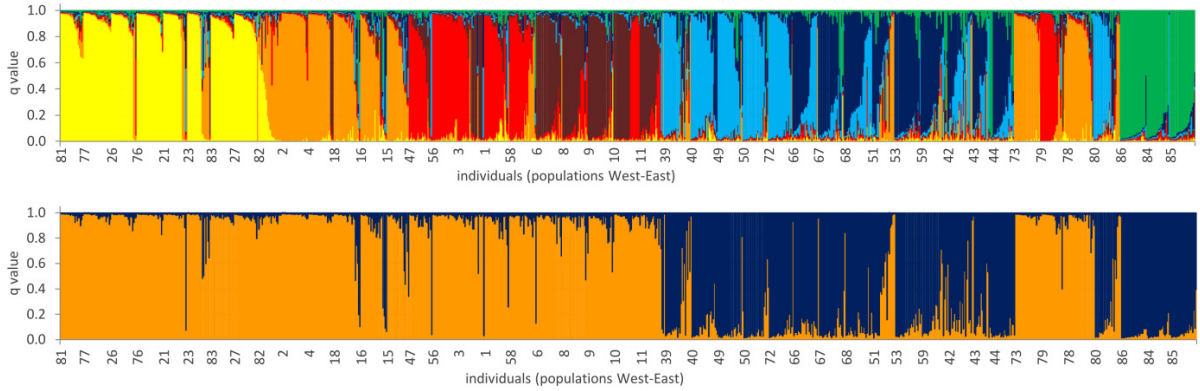

Table S 4 Simulation scenarios and assignment results for opposite group purebred and admixed category (groups explained in the text)

scenario	purebreds opposite group	regionally admixed
cluster 1 panmixia	0	0.013
cluster 2 panmixia	0	0.024
cluster 3 panmixia	0	0.023
cluster 4 panmixia	0	0.046
cluster 5 panmixia	0	0.054
cluster 6 panmixia	0	0.111
cluster 7 panmixia	0	0.059
group 1 panmixia	0	0.133
group 2 panmixia	0	0.123

Figure S 3 Number of observed translocation events versus the theoretical expectations according to the Poisson law.

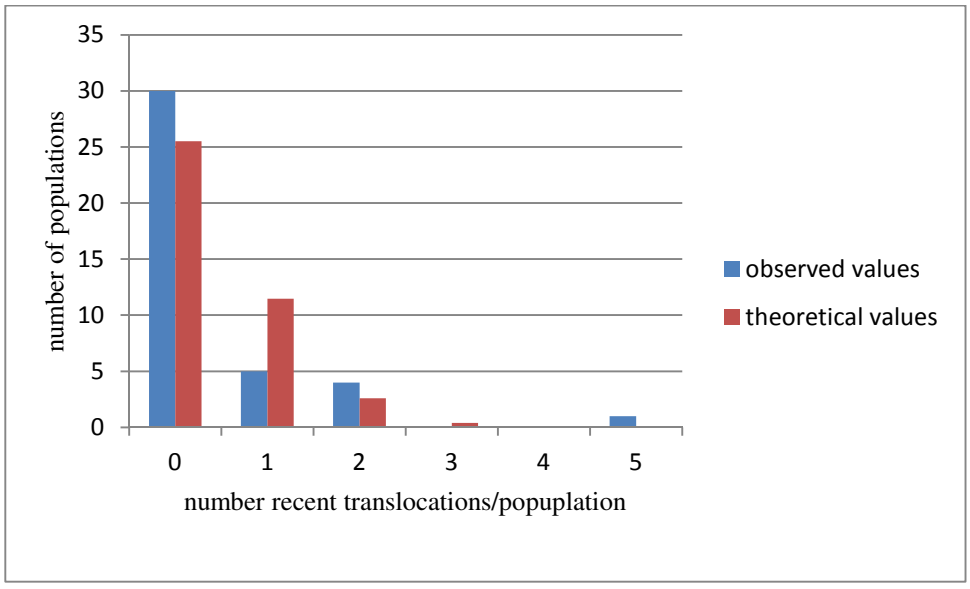

Figure S 4 Detections GENECLASS – detections STRUCTURE

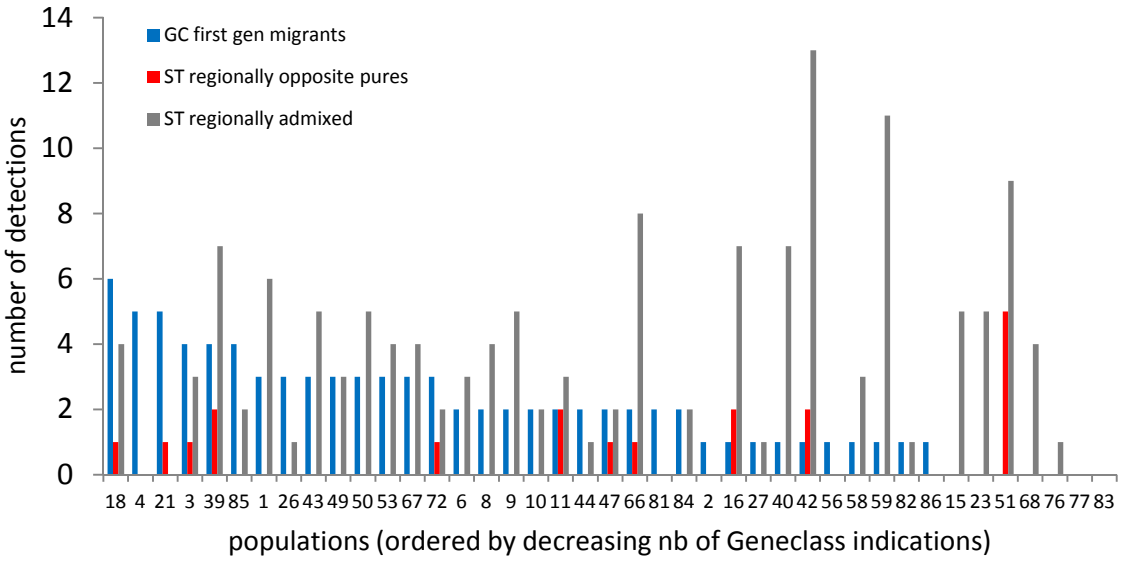

Figure S 5 Genetic composition of presumed natural and introduced material. Cluster colours are the same as in Fig. 6.

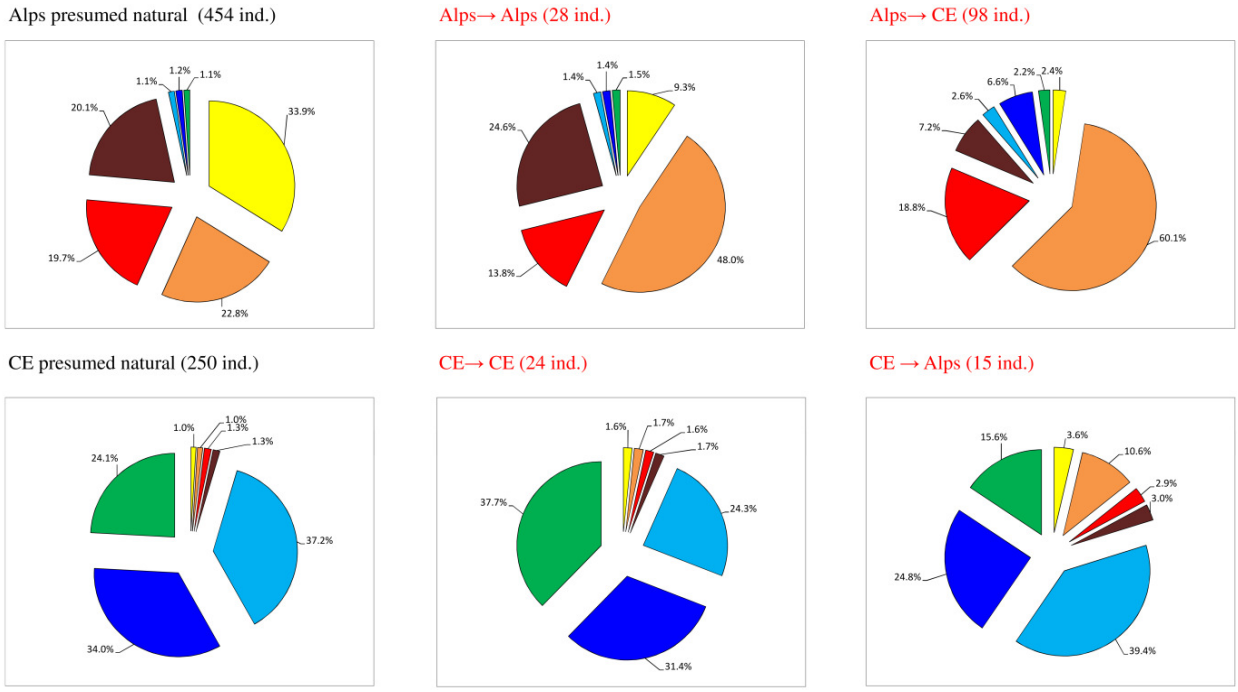

Table S 5 F_{IS} before and after systematic translocation removal

POP*	**g81**	**f81**	**g77**	**f77**	**g26**	**f26**	**g76**	**f76**	**g21**	**f21**	**g23**	**f23**	**g83**	**f83**	**g27**	**f27**	**g82**	**f82**
***F*IS per population**																		
bcLK25	-0.054	-0.057	0.002	0.002	0.156	0.199	0.007	0.007	0.12	-0.019	0.02	0	0.156	0.156	-0.062	-0.143	-0.078	-0.073
bcLK21	-0.01	-0.091	0.086	0.086	-0.097	-0.172	0.095	0.104	0.115	0.099	0.016	-0.09	-0.074	-0.074	0.078	0.012	-0.048	-0.085
bcLK22	-0.191	-0.218	0.1	0.1	0.126	0.094	0.019	-0.005	-0.055	-0.178	0.067	0.027	-0.003	-0.003	-0.217	-0.313	-0.121	-0.115
Ld30	0.222	0.259	-0.091	-0.091	-0.066	-0.186	-0.019	0.001	0.17	0.026	-0.02	0.018	-0.221	-0.221	-0.054	-0.188	0.25	0.224
Ld31	-0.12	-0.091	-0.25	-0.25	-0.108	-0.171	-0.011	-0.007	0.185	0.096	-0.013	-0.022	0.068	0.068	-0.228	-0.255	-0.018	0.009
Ld50	0.321	0.278	0.193	0.193	0.018	-0.266	0.016	-0.041	-0.011	-0.215	0.018	0.004	0.134	0.134	0.159	0.126	0.02	-0.098
bcLK18	-0.019	0.012	-0.209	-0.209	-0.202	-0.259	-0.048	-0.037	0.121	0.226	0.039	-0.04	0.059	0.059	-0.143	-0.222	0.317	0.299
Ld101	-0.054	-0.057	0.098	0.098	0.181	0	0.003	0.017	0.101	-0.032	-0.054	-0.008	0.056	0.056	0.068	0.12	-0.026	-0.025
Ld42	0.247	0.284	0.241	0.241	0.117	-0.179	-0.066	-0.029	0.164	-0.093	0.27	0.259	0.003	0.003	-0.082	-0.063	0.062	-0.157
Ld56	-0.087	-0.089	-0.124	-0.124	-0.058	-0.125	0.068	0.083	0.048	0.057	0.095	0.152	-0.166	-0.166	-0.123	-0.173	0.021	0.019
Ld58	0.246	0.215	-0.075	-0.075	0.027	-0.085	-0.002	0.012	0.169	0.121	0.246	0.197	0.049	0.049	0.077	0.13	0.127	0.164
bcLK26	-0.014	-0.016	-0.058	-0.058	0.113	0.097	0.043	0.048	0.107	0.086	0.183	0.167	0.162	0.162	0.086	0.113	0.021	0.025
Ld45	0.021	-0.053	0.165	0.165	0.162	0.17	-0.082	-0.073	0.194	-0.009	0.045	0.021	0.188	0.188	-0.004	-0.005	0.045	0.066
All	0.034	0.02	-0.002	-0.002	0.024	-0.062	0.005	0.01	0.111	0.019	0.07	0.05	0.029	0.029	-0.032	-0.066	0.046	0.029

POP*	**g2**	**f2**	**g4**	**f4**	**g18**	**f18**	**g16**	**f16**	**g15**	**f15**	**g47**	**f47**	**g56**	**f56**	**g3**	**f3**	**g1**	**f1**
***F*IS per population**																		
bcLK25	0.055	-0.003	0.072	-0.069	0.045	0.039	-0.059	-0.114	-0.079	-0.04	0.079	0.057	0.143	0.16	0.035	-0.036	0.046	0.027
bcLK21	-0.064	-0.095	-0.03	-0.094	0.062	0.018	0.14	-0.049	-0.024	-0.006	0.169	0.044	0.073	0.091	0.034	0.025	-0.026	-0.106
bcLK22	-0.205	-0.195	-0.01	-0.122	-0.035	-0.032	0.033	0.083	0.011	0.04	-0.061	-0.06	0.006	0.012	0.227	0.299	-0.198	-0.348
Ld30	-0.021	0	0.031	-0.205	-0.026	-0.211	0.013	0.061	-0.144	-0.19	0.213	0.345	-0.062	-0.077	0.083	0.015	-0.073	-0.092
Ld31	0.214	0.193	-0.122	-0.193	-0.147	-0.199	-0.03	0.006	-0.032	-0.08	0.021	-0.014	-0.025	0.005	0.107	0.128	0.065	0.112
Ld50	-0.096	-0.075	0.052	0.018	0.071	0.086	-0.026	-0.263	-0.224	-0.382	0.034	-0.003	-0.003	-0.02	0.18	0.02	0.132	0.081
bcLK18	0.019	-0.061	-0.078	-0.051	0.012	-0.078	0.069	-0.034	0.089	-0.026	0.075	0.111	0.079	0.056	0.261	0.164	0.133	0.125
Ld101	-0.045	-0.066	-0.077	-0.067	0.582	0.458	-0.177	-0.102	0.034	0.028	-0.053	-0.067	-0.116	-0.096	0.29	-0.014	-0.054	-0.054
Ld42	-0.136	-0.151	0.121	0.026	0.223	0.39	0.188	-0.077	-0.179	-0.151	0.145	0.034	-0.143	-0.151	0.096	-0.008	0.031	-0.02
Ld56	-0.085	-0.119	-0.114	-0.167	-0.007	-0.158	0.064	0	0.015	0.04	-0.148	-0.071	-0.156	-0.157	0.023	-0.032	0.156	0.081
Ld58	0.093	0.031	0.085	0.072	0.084	0.082	0.076	0.109	-0.042	-0.021	0.282	0.304	-0.007	-0.073	-0.032	-0.024	0.109	0.106
bcLK26	-0.056	-0.052	0.201	0.182	0.151	-0.009	0.093	-0.047	-0.167	-0.189	-0.064	-0.106	-0.016	-0.024	0.028	0.05	0.073	0.054
Ld45	-0.177	-0.164	0.019	0.072	0.054	-0.091	0.083	-0.06	0.063	0.107	-0.09	-0.221	-0.102	-0.083	0.428	0.434	-0.008	0.091
All	-0.032	-0.053	0.022	-0.03	0.058	-0.007	0.042	-0.03	-0.051	-0.066	0.056	0.032	-0.011	-0.014	0.125	0.089	0.036	0.014

* g: global sample, f: final sample after removal; Fis was not recalculated if >50% of the individuals had been removed (populations 42, 51)

POP F_{IS} per population	g58	f58	g6	f6	g8	f8	g9	f9	g10	f10	g11	f11	g79	g39	f39	g40	f40
bcLK25	-0.006	-0.032	-0.02	-0.038	0.065	-0.014	-0.048	-0.036	-0.083	-0.164	0.08	0.016	0.011	-0.087	-0.025	0.011	-0.082
bcLK21	0.031	0.016	0.064	0.017	-0.146	-0.168	-0.184	-0.195	0.138	0.084	0.243	0.327	0.282	0.038	-0.036	-0.16	-0.117
bcLK22	-0.11	-0.106	-0.008	-0.027	-0.072	-0.059	-0.051	-0.046	0.254	0.297	-0.017	-0.109	-0.072	-0.034	-0.162	0.09	0.036
Ld30	-0.035	-0.056	0.07	0.094	0.025	0.053	-0.064	-0.148	0.246	0.255	0.036	-0.061	0.254	0.062	0.26	0.1	0.05
Ld31	-0.008	0.04	0.085	0.027	0.049	-0.021	-0.044	-0.118	0.179	0.133	0.111	0.262	-0.002	-0.129	-0.309	0.111	0.082
Ld50	0.065	0.085	0.025	0.007	-0.184	-0.152	0.088	-0.013	0.009	0.043	0.087	0.202	0.14	0.169	0.12	-0.064	-0.113
bcLK18	-0.235	-0.226	0.134	0.203	0.135	0.078	0.203	0.084	0.007	0.007	-0.023	-0.054	0.12	0.018	-0.108	0.148	0.183
Ld101	0.363	0.431	-0.04	-0.027	-0.023	0	-0.128	-0.172	-0.022	-0.026	0.25	0.474	0.29	0.108	0.184	0.108	0.138
Ld42	0.142	-0.109	0.023	-0.027	0.217	0.227	0.366	0.328	0.018	-0.063	0.16	0.018	-0.139	0.108	-0.013	-0.024	-0.167
Ld56	-0.001	-0.007	-0.074	-0.088	-0.219	-0.204	0.06	-0.047	-0.072	-0.061	0.162	0.136	0.196	0.01	-0.068	0.19	0.193
Ld58	-0.004	0.026	-0.052	-0.112	0.051	0.054	0.067	-0.063	-0.138	-0.138	0.048	-0.074	0.321	0.17	0.287	0.048	0.051
bcLK26	0.203	0.26	-0.042	-0.079	0.035	-0.057	0.032	0.074	-0.048	-0.058	0.037	0.089	-0.051	0.448	0.516	0.208	0.154
Ld45	-0.204	-0.195	-0.009	-0.026	-0.037	-0.071	0.229	0.118	0.287	0.296	-0.008	-0.135	0.025	0.02	-0.091	0.041	0.002
All	-0.001	-0.002	0.015	-0.005	-0.006	-0.03	0.038	-0.019	0.064	0.052	0.079	0.053	0.1	0.073	0.047	0.065	0.036

POP F_{IS} per population	g49	f49	g50	f50	g72	F72	g66	f66	g67	f67	g68	f68	g51	g53	f53	g59	f59
bcLK25	-0.153	-0.156	-0.086	-0.039	-0.011	-0.062	0.109	0.036	-0.065	-0.078	-0.12	-0.133	-0.042	-0.047	-0.015	0.02	0.023
bcLK21	0.066	-0.012	0.036	-0.043	0.272	0.229	-0.011	0.022	-0.027	-0.037	0.002	0.056	-0.084	-0.106	-0.078	0.055	0.022
bcLK22	-0.098	-0.082	0.003	-0.021	-0.038	-0.106	-0.073	-0.139	0.148	0.224	0.048	0.082	-0.006	-0.011	-0.07	-0.07	-0.068
Ld30	-0.121	-0.129	0.077	-0.021	-0.072	-0.114	-0.175	-0.148	-0.082	-0.103	0.075	0.021	0.029	0.228	0.27	-0.07	-0.134
Ld31	-0.094	-0.095	0.045	0.092	0.11	0.13	0.19	0.044	0.061	0.067	0.051	0.081	-0.021	0.062	-0.071	-0.006	0.08
Ld50	-0.004	0.025	0.058	0.015	0.173	0.16	0.225	0.366	0.031	-0.012	0.199	0.199	0.011	0.189	0.223	0.1	-0.099
bcLK18	0.073	0.02	0.112	0.091	0.019	0.018	-0.034	-0.12	0.035	-0.011	0.01	0.036	-0.025	-0.044	0.002	-0.001	-0.101
Ld101	0.012	0.089	0.021	-0.022	0.017	0.142	-0.138	-0.036	0.429	0.521	-0.162	-0.145	-0.034	0.066	0.084	0.11	0.262
Ld42	0.063	-0.006	0.129	0.129	-0.071	0.015	0.284	0.392	0.01	-0.068	0.207	0.171	-0.106	0.134	0.214	0.241	0.273
Ld56	0.106	0.098	-0.099	-0.244	0.203	0.216	0.292	0.514	0.36	0.202	0.027	0.004	0.136	0.179	0.252	0.153	0.098
Ld58	0.174	0.163	0.002	0.026	0.08	0.008	0.021	0.077	0.019	-0.026	-0.052	-0.032	0.013	0.074	0.133	0.08	-0.021
bcLK26	0.554	0.524	0.299	0.293	0.411	0.479	0.345	0.568	0.114	0.005	0.232	0.277	0.084	0.08	0.05	0.19	0.282
Ld45	-0.193	-0.291	0.146	0.269	-0.093	-0.13	0.108	0.2	-0.026	-0.011	-0.012	0.03	0.094	0.011	0.121	0.181	0.287
All	0.037	0.016	0.058	0.043	0.088	0.077	0.098	0.149	0.071	0.04	0.046	0.059	0.009	0.062	0.085	0.073	0.063

* g: global sample, f: final sample after removal; Fis was not recalculated if >50% of the individuals had been removed (populations 42, 51)

POP Fis per population	g78	g80	g42	g43	f43	g44	f44	g86	f86	g84	f84	g85	f85	g73
bcLK25	0.004	0.102	-0.075	0.087	0.06	0.029	-0.058	-0.069	-0.07	0.004	0.015	-0.006	-0.028	0.013
bcLK21	0.278	0.021	0.034	0.046	0.03	-0.08	-0.117	-0.115	-0.139	0.287	0.259	-0.038	-0.015	0.085
bcLK22	0.001	0.121	0.093	0.111	0.058	-0.01	0.005	0.061	0.07	-0.128	-0.146	-0.041	-0.092	-0.045
Ld30	-0.042	0.153	0.021	0.155	-0.174	0.279	0.375	0.204	0.239	0.185	0.353	0.192	0.148	-0.062
Ld31	-0.143	-0.062	0.064	0.049	-0.052	-0.073	-0.06	0.302	0.258	0.253	0.259	0.217	0.127	-0.04
Ld50	-0.044	0.12	0.233	0.155	-0.025	0.042	0.002	0.125	0.141	-0.107	-0.094	0.099	0.131	-0.087
bcLK18	0.095	0.2	-0.027	0.111	0.169	-0.013	-0.055	0.082	0.065	0.28	0.337	0.08	0.197	-0.084
Ld101	-0.228	0.082	0.38	0.092	-0.213	-0.157	-0.25	-0.136	-0.143	0.135	0	-0.038	0.005	0.1
Ld42	-0.029	-0.015	0.104	0.243	0.125	-0.195	-0.278	0.079	0.032	-0.12	-0.164	0.014	0.059	0.093
Ld56	-0.045	0.052	0.051	0.382	0.275	0.036	0.013	0.248	0.11	0.183	0.226	0.085	0.096	0.01
Ld58	0.128	0.058	-0.003	0.03	-0.038	-0.043	-0.037	0.018	-0.04	-0.017	-0.041	0.028	0.104	0.208
bcLK26	0.026	0.283	0.154	0.111	0.241	-0.045	-0.051	-0.118	-0.115	0.331	0.212	-0.015	-0.021	0.016
Ld45	0.006	-0.017	0.041	0.195	0.223	-0.056	-0.098	0.068	-0.162	0.432	0.424	0.207	0.137	0.121
All	0.007	0.089	0.068	0.134	0.06	-0.015	-0.033	0.056	0.03	0.137	0.139	0.062	0.062	0.023

* g: global sample, f: final sample after removal; Fis was not recalculated if >50% of the individuals had been removed (populations 42, 51)

CHAPTER 5:

Millennial scale flexibility of European larch populations

Introduction

High resolution records documenting climate history open new perspectives for investigating forests dynamics (Fletcher *et al.* 2010; Sánchez Goñi & Harrison 2010). Climatic oscillations and coincident forest development have been analyzed for the last and current interglacial (e.g. Berglund *et al.* 1996; Drescher-Schneider 2000; Zagwijn 1996). In contrast, short-term climate events, i.e. changes within <200 years (Sánchez Goñi & Harrison 2010), and their influence on vegetation cover are still understudied, due to the limited resolution of the pollen records. The situation has started to change with the availability of the high resolution Greenland ice-core record NGRIP (e.g. Andersen *et al.* 2004; Johnsen *et al.* 1992; Wolff *et al.* 2010) and marine cores (e.g. Fletcher *et al.* 2010; Roucoux *et al.* 2001; Sánchez Goñi *et al.* 2008; Sánchez Goñi *et al.* 2002) registering numerous short-term climate events during the last glacial period. Vegetational responses to these events have been quasi-instantaneous and very heterogeneous. While the general history of European forests has now started to be investigated at this resolution for the period of the last glacial (Fletcher *et al.* 2010; Pini *et al.* 2010; Sümegi *et al.* 2013), it seems timely to study the flexibility of individual tree taxa towards short-term climate oscillations as this can assist in preparing forests for future climate change.

The most significant climate variation of the Quaternary is the alternation of glacial and interglacial climates that occurred regularly with an approximately 100 ka periodicity since ~800 ka. This variation is explained by external orbital parameters leading to changes in latitudinal and seasonal patterns of insolation modulated by internal factors such as ice-sheet extent or greenhouse gas concentration. Short-term changes are superimposed on long-term changes and occur at the millennial scale and with irregular periodicity (Sánchez Goñi & Harrison 2010; Wolff *et al.* 2010). They are particularly striking during the last glacial between 73.5 and 14.7 ka. There are two types of such rapid climate events. First, Dansgaard-Oeschger (D-O) events (Dansgaard *et al.* 1984) correspond to abrupt warming registered in the Greenland ice-core record (e.g. Andersen *et al.* 2004; Johnsen *et al.* 1992; Wolff *et al.* 2010). Second, Heinrich events (HE) (Heinrich 1988) represent rapid cooling resulting in ice-rafted debris (IRD) found in marine cores of the North Atlantic. The standard reference ice core is the Northern Greenland Ice Core Project (NGRIP; North GRIP Members 2004) encompassing the period from the last interglacial through the last glacial into the present (Fig. 10). It shows 25 D-O cycles, of which 18 are in the last glacial. The typical D-O event is a very sharp warming event occuring within a few decades (Wolff *et al.* 2010).

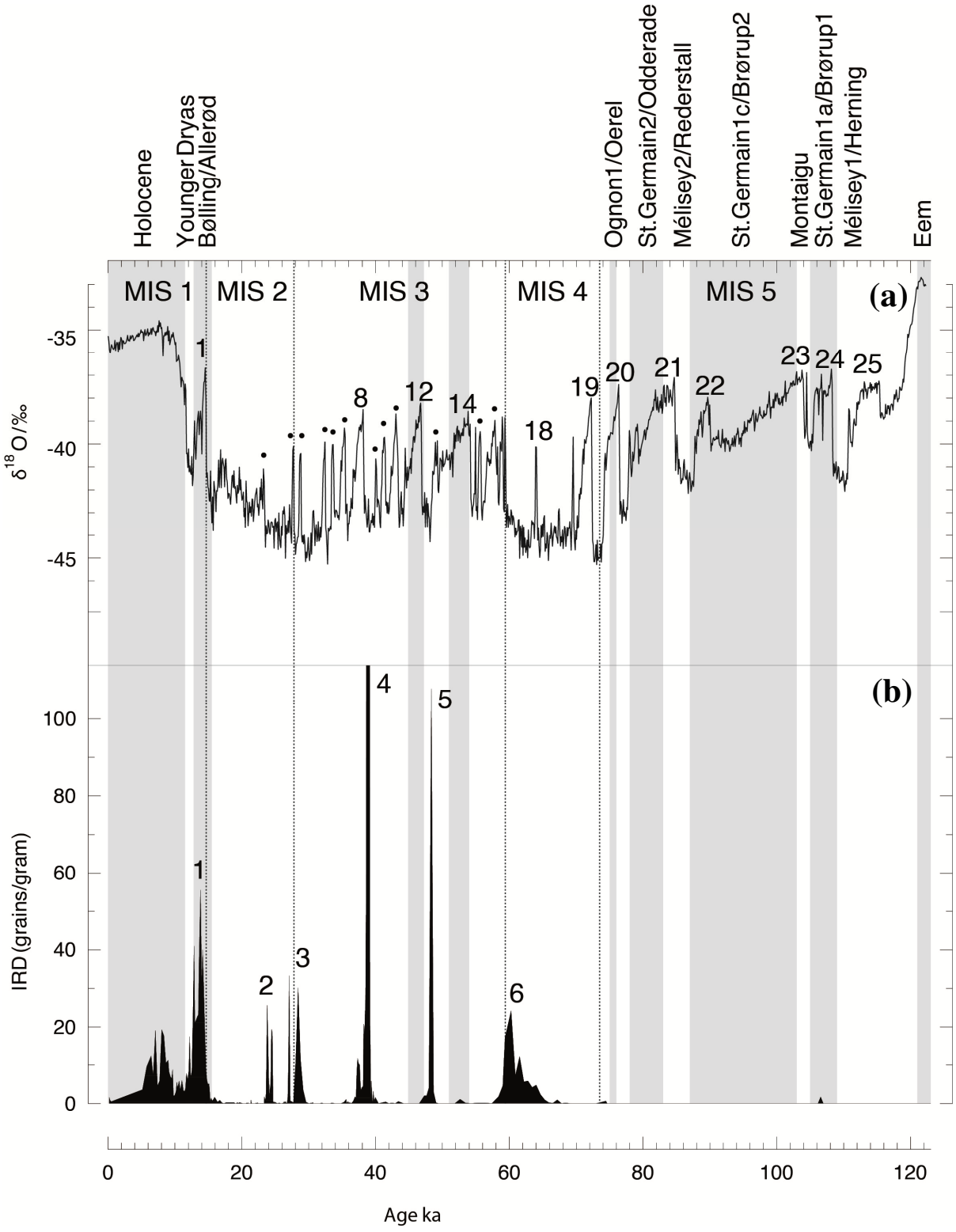

Figure 10 Abrupt climate events since 123 ka (a) Dansgaard-Oeschger events in the NGRIP ice core [after Wolff *et al.* 2010]; (b) Heinrich Events in the MD99-2331 core from the North Atlantic, (data provided by M. Sánchez Goñi, published in Sánchez Goñi *et al.* 2008); above: regional biostratigraphic units (Woillard *et al.* 1978/Behre *et al.* 1986, 1989) cited in the text. Grey shaded bars indicate periods of important forest advances at latitudes >40°N.

Duration and intensity of D-O events were variable. During MIS 5 they were particularly long. During MIS 4 occurred the strongest warming of the last glacial cycle (D-O 19, 16°C, Landais *et al.* 2004). Apart from this event other events during MIS 4 and MIS 2 were relatively weak, whereas they were more pronounced during MIS 3. The final D-O event is generally considered to be synchronous with the Bølling-Allerød interstadial at the beginning of MIS 1 (~14.7 – 12.8 ka). Ice-core derived temperature estimates for different D-O events range between 8°C and 16°C. Heinrich events occurred during MIS 2, 3 and 4. They seem to have been particularly severe during MIS 2 and MIS 4 when global ice volume was maximal and insolation was minimal. For the Holocene (since ~11.7 ka) there are no D-O or Heinrich events registered. However, there were some rapid climate changes in the early Holocene, the most prominent being the 8.2 ka cold event likely caused by the final collapse of the Laurentide ice sheet of northeastern North America (Ellison *et al.* 2006). Climate changes documented in the ice cores and marine cores (including marine pollen records) from the North Atlantic can be correlated with continental pollen records to reassess vegetational development documented based on regional biostratigraphic units (Fig. 10).

In Europe, the availability of large amounts of fossil data has enabled detailed reconstructions of late glacial and Holocene tree histories. In most of these investigations, paleontological data have been combined with modern genetic data, e.g. *Quercus*, *Fagus sylvatica*, *Pinus sylvestris, Abies alba, Picea abies* (Cheddadi *et al.* 2006; Falush *et al.* 2003; Liepelt *et al.* 2009; Magri *et al.* 2006; Petit *et al.* 2002; Tollefsrud *et al.* 2008). To date, however, the impact of past abrupt climate events on tree histories has not been investigated. Yet, such knowledge could be an important foundation to understand trees flexibility towards future climate change.

Pioneer tree species could represent useful models for such studies as their response to climate should be particularly rapid due to their high rate of spread and modest soil and temperature requirements. However, pollen in these taxa is typically dispersed at long distances and its detection, at least at low concentration, cannot therefore be used to testify for the presence of a species in a given locality. Interestingly, European larch (*Larix decidua* Mill.), a pioneer tree species, does not disperse very well its pollen (e.g. Jankovská 2007; Pelánková & Chytrý 2009; Sjögren *et al.* 2010; Sjögren *et al.* 2008a; Sjögren *et al.* 2008b). Furthermore, its needles are well preserved as macrofossils. To date, *Larix* has not been the subject of recent historical studies. Its modern distribution range is disjunct and comprises four distinct areas, the Alps, the Sudetes Mts, the Tatra Mts, and central Poland. In addition, it

has a more sporadic distribution in the eastern and southern Carpathians and in the Bihar mountains of Romania (Rubner 1953). Altitudinal distribution varies widely. The Central Alps with most prominent larch occurrences are definitely continental whereas occurrences in the northern Alps or in the Sudetes Mountains differ from the continental climate type (McComb 1955; Rubner 1953). However, all occurrences are situated in montane to subalpine regions except a few populations located in the Polish lowlands.

This study focuses on a range-wide reconstruction of the history of the European larch during the last 130,000 years based on a new comprehensive fossil compilation. The issues we address are (i) the chronological documentation of range changes since the last interglacial, (ii) the identification of the consequences of D-O events and Heinrich events on larch populations, (iii) the identification of LGM refugia and postglacial migration pathways, and (iv) the characterization of the origin (natural versus anthropogenic) of changes in larch geographic distribution and abundance.

Materials and methods

Data compilation

Pollen, macrofossil, stomata and charcoal data for *Larix* at the European scale are compiled over the last 130 ka. For 130 – 19 ka data are newly assembled from literature. During literature compilation we first evaluated if the investigator has been able to recognize *Larix* pollen. This is necessary because *Larix* pollen is hard to identify and has remained unnoticed in some earlier investigations due to its similarity with spores.

For the last 19 ka years macrofossil, stomata, charcoal and pollen data from literature are combined with pollen data from the Alpine Palynological Database (ALPADABA), the Czech Quaternary Palynological database (PALYCZ, http://botany.natur.cuni.cz/palycz/, Kuneš *et al.* 2009) and the European pollen database (EPD), which has recently benefitted from new chronologies (Giesecke *et al.* 2013). Data are compiled in this sequence from the corresponding databases. In addition stomata records stored in the EPD (http://www.europeanpollendatabase.net/) are included.

Pollen productivity, dispersion, identification

As pointed out before, pollen productivity and dispersal of *Larix* are limited, suggesting that even single pollen counts can indicate the local presence of the species. However, we cannot completely exclude that scarce single counts result from long-distance wind-transport, especially at the high altitudinal belts of high mountain systems that may be characterized by strong winds. After carefully checking all sequences one by one, we decided to include single pollen data as an indication for the local presence of the species, unless the findings stayed exceptional. Correspondingly, we documented for each record if the findings were scattered or more or less continuous and transferred the information on the maps.

Chronological mapping

After compilation all data are assigned to successive time intervals representing biostratigraphic units (130 -11.7 ka). For the Holocene (since 11.5 ka) 1000 year intervals are used. For each time interval positive and negative evidence given by pollen, macrofossils, stomata and charcoals are mapped using ArcGIS 9.3 (ESRI 2009).

Results and discussion

Data compilation

1026 fossil sites of which 355 include *Larix* evidence have been compiled (Fig. 11, Table S 6 – S 13, List 1, List 2, List 3, Supporting information). This compilation is based on newly assembled sites from the literature (207 sites including 169 *Larix* sites), ALPADABA sites (156/105), PALYCZ sites (114/30) and complementary EPD sites (549/51).

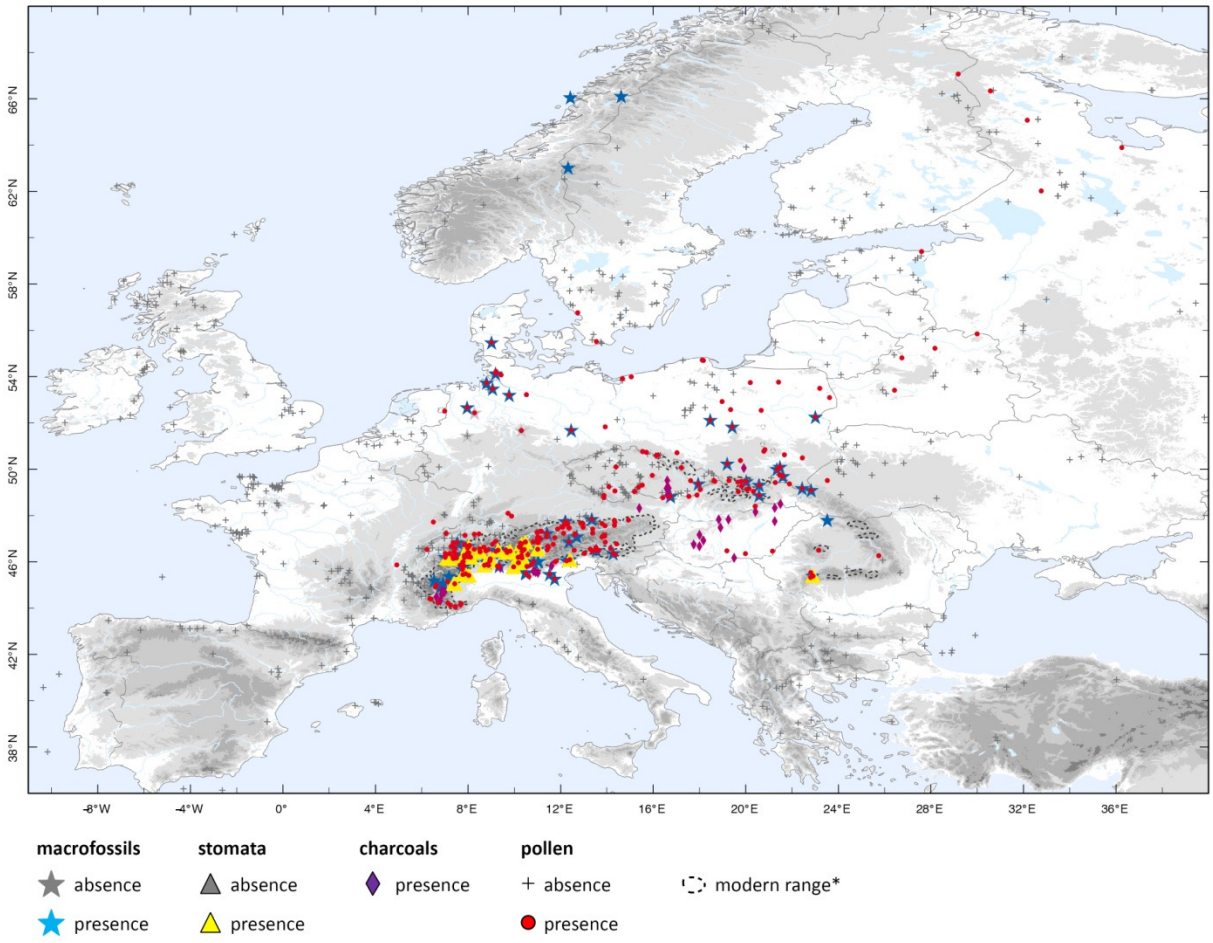

Figure 11 Map of the 1026 fossil sites investigated in this study

* modern distribution data compiled by E. Welk, AG Chorology, Geobotany Department, University Halle, based on map 21b in Meusel *et al.* (1965); modified in central Poland

MIS 5 (~130 – 73.5 ka)

For MIS 5, Alpine and north-central European profiles are studied based on the regional biostratigraphic units that are correlated to the NGRIP chronology to identify climate events from the northern hemisphere that should be isochronous across Europe (Fig. 10).

Last interglacial *sensu stricto* (~130 – 112 ka, Sánchez Goñi *et al.* 2012)

For the last interglacial (Eemian) 41 fossil sites are found. 14 of them include scattered *Larix* pollen and macrofossils in the Alpine and the north-central European regions (Fig. 12, Table S 6 Supporting information). Larch fossils are only found at the transitions of this interglacial. Larch likely had a wider distribution range than today as fossils not only occurred in the Alpine region but also in the north-central European lowlands. In addition, larch can be expected to have persisted in the Carpathian region. However this cannot be proven due to the lack of fossil sites.

In the Alpine region the first evidence is found at the transition from the penultimate glacial (Rissian) to the last interglacial (Eemian). Fossils occur for instance in Mondsee (540 m a.s.l.) in the northern Alpine foreland and in Lake Fimon (23 m a.s.l.) in the southern Alpine foreland (Drescher-Schneider 2000; Pini *et al.* 2010). At this time *Larix* was growing in light forests dominated by *Pinus* and *Betula*. These pioneer formations were replaced by rapidly expanding thermophilous trees favoured by climatic warming. All *Larix* fossil sites of the Alpine region are located below 700 m; the species disappeared from these sites during the temperate part of the Eemian. The absence of *Larix* fossils during the temperate phases could be due to the upward shift of the species and the lack of data at higher altitudes, by analogy with the temperate phase of the current interglacial (Holocene), during which such an upward shift could be traced with fossils (see below).

New evidence from Mondsee and Lake Fimon as well as from other sites of the northern Alpine foreland, for instance Jammertal in Southern Germany (Müller 2000) or Gondiswil in Switzerland (Wegmüller 1992), is found at the transition to the first last glacial stadial (Mélisey I) during climatic cooling at ~112 ka (Sánchez Goñi *et al.* 2012).

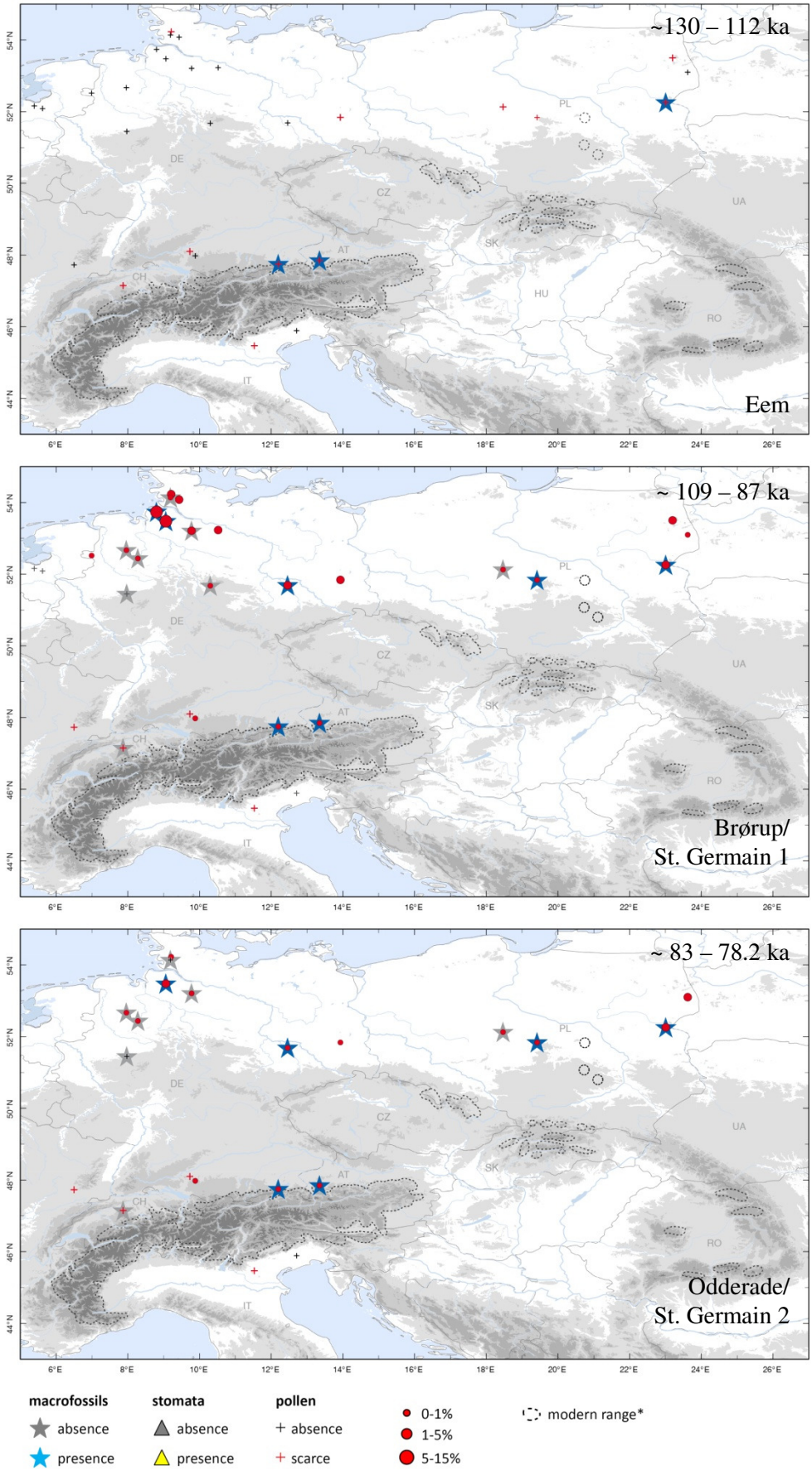

Figure 12 Fossil distribution of larch from 130 – 78.2 ka

At the transition to Mélisey I *Larix*, together with *Pinus* and *Picea*, replaced less cold-tolerant deciduous trees. For the Alpine region, it has been proposed that these coniferous forests prevailed during about 5 ka and were then replaced by a tundra-like vegetation with prevailing non-arboreal components (Müller & Sánchez Goñi 2007). The Mondsee record demonstrates that small groups of trees including *Larix* and other cold-tolerant arboreal species (*Picea, Pinus, Pinus cembra, Salix, Juniperus, Alnus viridis*) persisted during the Mélisey 1 period at mid altitudes (Drescher-Schneider 2000).

In the north-central European lowlands *Larix* pollen was found at the transition from the penultimate glacial (Saalian) in one site located in central Poland (Horoszki Duze, 180 m a.s.l., Granoszewski 2003). As in the Alpine region, evidence during the temperate phases is lacking. The species is found again at the transition to the first last glacial stadial (Herning) in several Polish and northern German sites (e.g. Erd 1973; Granoszewski 2003; Menke & Tynni 1984) in communities dominated by *Pinus* and *Betula*.

The first last glacial stadial in northwestern and central Europe, which is unfortunately poorly documented, was characterized by tundra environments with predominating steppic taxa (for review see Caspers & Freund 1997). Scarce *Larix* pollen evidence from different locations together with other arboreal components like *Pinus, Betula, Betula nana, Juniperus* (Behre *et al.* 2005; Behre & Lade 1986; Erd 1973; Granoszewski 2003; Menke & Tynni 1984) suggest scattered occurrence of larch but species persistence cannot be proven conclusively due to insufficient resolution of the profiles.

First early Weichselian interstadial (~109 – 87 ka, Müller & Sanchez Goñi 2007)

The forests corresponding to the first last glacial interstadial can be studied in 42 fossil sites including 28 with *Larix* evidence (Fig. 12 , Table S 7, Supporting information). Larch fossils are found in the Alpine region and the north-central European lowlands and also in the Vosges Mountains. In the north-central European lowlands fossil abundance indicates a distributional maximum of larch.

In the Alpine region larch pollen and macrofossils are found in the northern and southern Alpine foreland, e.g. in Füramoos (Germany), Mondsee (Austria) and Lake Fimon (Italy) (Drescher-Schneider 2000; Müller 2001; Müller *et al.* 2003; Pini *et al.* 2010). Larch was a component of coniferous formations largely dominated by *Picea* and *Pinus*. Generally evidence was strongest during early successional stages and during climatic cooling at the

transition to the subsequent stadial (Mélisey 2) suggesting an upward shift during the climatically most favourable times (e.g. Mondsee, Samerberg and Gondiswill, Drescher-Schneider 2000; Grüger 1979; Wegmüller 1992). In some sites (e.g. Füramoos and Lake Fimon, Müller 2001; Müller *et al.* 2003; Pini *et al.* 2010), *Larix* was clearly favoured by the Montaigu event, an abrupt cooling episode in the second third of St. Germain 1, St. Germain 1b, between 105 ka and 103 ka (Müller & Sánchez Goñi 2007; Woillard 1978). At La Grande Pile in the Vosges Mountains, a massif that lies outside the current species range, the same trend is observed (De Beaulieu & Reille 1992a).

In the north-central European lowlands the presence of *Larix* is evidenced in multiple sites in Germany and Poland during this first last glacial interstadial (Brørup). Macrofossils are found at several places and pollen percentages were conspicuously high for larch with maximum values up to 18% (e.g. Osterwanna, Oerel and Keller, Behre 1974; Behre *et al.* 2005; Behre & Lade 1986; Menke 1970) indicating that it was a major component of boreal forests in which it occurred together with *Picea*, *Pinus* and *Betula*. Colonization of the Central European lowlands by larch started from the southeast and proceeded to the northwest. After its first establishment, the species was favoured by a cold event located within the Brørup interstadial, corresponding to the Montaigu event, which affected competing species (e.g. Caspers 1997; Caspers & Freund 1997; Menke & Tynni 1984). After this cold event, *Larix* reached its maximal distribution in the second half of the Brørup.

At the transition to the second stadial (Rederstall) *Larix* declined, probably as a consequence of increased continentality (e.g. Caspers & Freund 1997; Menke & Tynni 1984; Ricken & Grüger 1988). Considering the whole last interglacial/glacial interval the period between 105 ka (beginning of Montaigu event) and 100 ka (end of Brørup) seems to be the period of maximal larch distribution in the north-central European lowlands.

In the second early Weichselian stadial (Mélisey 2/Rederstall) tundra-like formations prevailed in the Alpine and the north-central European region. However, there is still some pollen and macrofossil evidence of *Larix* found in several sites, in particular in the Alpine region, indicating that scattered trees still occurred together with other cold-tolerant trees such as *Picea, Pinus, Pinus cembra*, *Betula, Juniperus* (e.g. Drescher-Schneider 2000; Erd 1973; Hahne *et al.* 1994b; Müller 2000, 2001). It seems likely that in the Alpine region boreal trees did not only occur in the analysed intermediate altitudinal sites but that they also colonized the lowlands and built mosaic landscapes with steppic taxa.

Second Weichselian interstadial (~83 – 78.2 ka, Sanchez Goñi pers. comm.)

The second early Weichselian interstadial (St. Germain 2/Odderade) can be studied in 34 sites, 20 of which contained *Larix* evidence (Fig. 12, Table S 8, Supporting information). The fossil distribution and the role of larch in the coniferous forests of the Alpine and north-central European region are similar to those from the first interstadial. Some differences can be detected in the vegetational succession and in the reduced pollen and macrofossil representation in the central European lowlands, pointing to a weaker abundance than during the Brørup (e.g. Behre *et al.* 2005; Behre & Lade 1986; Caspers & Freund 1997; Menke & Tynni 1984).

In north-central Europe the second interstadial was followed by the final decline of early Weichselian woodland and the spread of tundra and steppe vegetation. In contrast, in the Alpine region there was another forested interstadial (Ognon 1 ~ DO 20 ~ 76.4 – 75.5 ka, Wolff *et al.* 2010) that was characterized by conspicuous *Larix* advances observed at several places (Drescher-Schneider 2000; Grüger 1979; Grüger & Schreiner 1993; Müller 2001; Müller *et al.* 2003; Wegmüller 1992; Welten 1982a). In Gondiswil for instance pollen values reached 20% (Wegmüller 1992). After the Ognon 1 interstadial forest vegetation was finally replaced by steppic communities, including in the Alpine region.

MIS 4 (~73.5 – 59.4 ka)

For MIS 4 there are only few fossil sites (Fig. 13, Table S 9, Supporting information). Scattered *Larix* pollen is found north (Beerenmösli, Wegmüller 1992) and south of the Alps (Pini *et al.* 2010) as well as in central Poland (Komar *et al.* 2009) indicating that the species should have persisted in the Alpine region as well as in Central Europe.

At the sites where *Larix* was found it occurred together with prevailing tundra components pointing to small tree populations growing within open vegetation. In Füramoos north of the Alps (Müller 2001; Müller *et al.* 2003), *Larix* and other tree taxa disappeared during MIS 4, indicating harsh climate conditions. Considering the unfavourable climatic character of MIS 4, due to low insolation and the Heinrich 6 event, this stage can be assumed to have had dramatic consequences for larch populations in all parts of its range by causing major extinctions and strongly restricting population size.

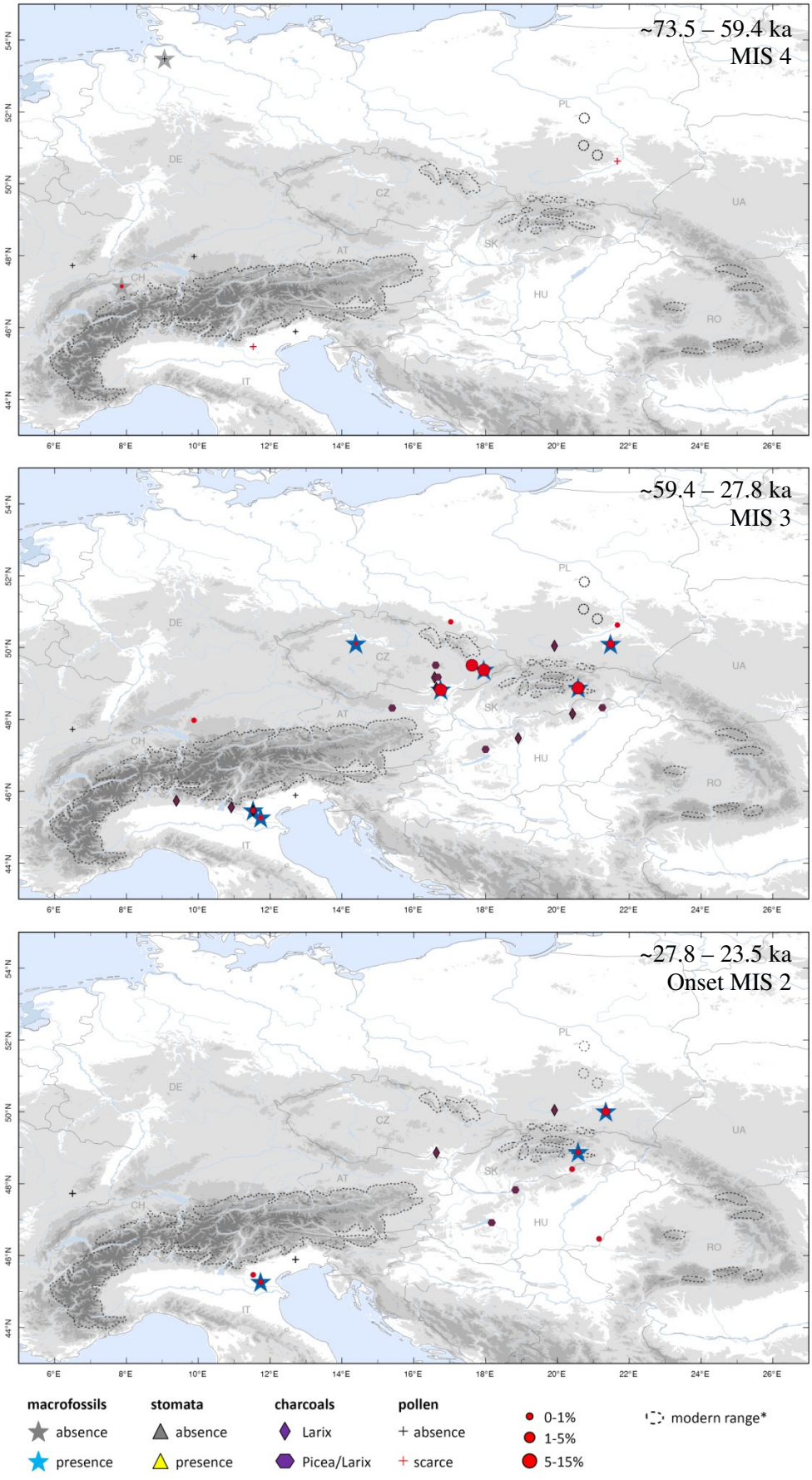

Figure 13 Fossil distribution of larch from 73.5 – 23.5 ka

MIS 3 (~59.4 - 27.8 ka)

For MIS 3 fossil sites are more numerous (Fig 13, Table S 10, Supporting information) and include *Larix* pollen, macrofossils and charcoals. Fossil distribution covers a wide latitudinal and longitudinal range in particular in the Carpathian region and in the Pannonian basin.

In the Alpine region different aspects of larch history are documented in the northern Alpine site Füramoos and in the southern Alpine sites of Lake Fimon and Lago della Costa (Kaltenrieder *et al.* 2009; Müller *et al.* 2003; Pini *et al.* 2010; Wick 2006). In the southern sites macrofossils corroborate pollen evidence, pointing to local persistence of larch during MIS 3 within a mosaic of boreal forests and steppes. In addition, individually dated charcoals were found at the southern Alpine fringe in the provinces of Verona and Como (Maspero 1996) between ~ 46 ka and 36 ka. This suggests a relatively wide species distribution range in the southern Alpine foreland and would fit well with studies reporting alternating episodes of spread of boreal and temperate trees (Canali *et al.* 2007; Drescher-Schneider *et al.* 2007; Pini *et al.* 2009; Preusser & Degering 2007). Inferences on *Larix* response to D-O events can be made on the basis of the Füramoos pollen record (Müller 2001; Müller *et al.* 2003). This record reveals that *Larix* together with *Betula* was favoured by D-O events 14 and 12, which date back to 54.2 ka and 46.8 ka, respectively (Wolff *et al.* 2010). In contrast, earlier D-O events 17 (~59.4 ka) and 16 (~58.2 ka) did not elicit such a response. This is consistent with a recent study demonstrating that at latitudes above 40°N D-O 12 and 14 triggered the most prominent forest advances (Sánchez Goñi *et al.* 2008). In Lake Fimon individual D-O events cannot be discerned as general forest development was strong due to orographic rainfall. However, pollen curves do suggest that a cluster of D-O events including D-O 12 and D-O 14 favoured *Larix* as well as other taxa (*Picea, Betula* and *Alnus*).

For the Carpathian region persistence of *Larix* during MIS 3 is proven by the pollen sequences of Šafárka (600 m a.s.l.) and (in part) of Jablůnka (350 m a.s.l.) stemming from two well protected intermountain basins of the Western Carpathians (Jankovská 2003; Jankovská *et al.* 2002; Jankovská & Pokorny 2008). Pollen records supported by macrofossil show values up to 20% in the bottom part of the Šafárka sequence and indicate predominance of larch together with *Betula* and *Pinus cembra* followed by predominance of *Picea* since ~30 ka (Kuneš *et al.* 2008). The pollen assemblages from both sites have been compared to modern analogues from southern Siberia. Results have revealed that the Western Carpathians had been covered by varying vegetation complexes encompassing taiga forests, hemiboreal forests, forest tundra, forest steppes, tundra or steppes (Kuneš *et al.* 2008). Responses to D-O

events cannot be studied as resolution and dating are insufficient. The complementary information given by single dated pollen probes, macrofossils and charcoals from between ~55 ka and 28 ka (e.g. Damblon & Haesaerts 1997; Damblon *et al.* 1996; Erd 1973; Geyh *et al.* 1969; Komar *et al.* 2009; Krolopp 1977; Mamakowa & Starkel 1974; Musil 2003; Opravil 1994; Rybníčková & Rybníček 1991; Willis & van Andel 2004) suggest great abundance of larch in the surroundings of the Carpathian Mountains and in the Pannonian Plain, within a mosaic of more or less open forest tundra, forest steppe or small forests.

MIS 2 (~27.8 – 14.7 ka)

Pre-LGM and LGM period

For the part of MIS 2 preceding the LGM (~27.8 – 23.5 ka, Fig. 13) and the LGM (~23.5 – 19 ka, Fig. 14) (Mix *et al.* 2001; Sánchez Goñi & Harrison 2010), there are only few fossil sites. However, they clearly demonstrate the persistence of *Larix* in the Alpine and the Carpathian region as well as in the Pannonian basin.

Two *Larix* sites were studied in the Alpine region: Lake Fimon and Lago della Costa (Kaltenrieder *et al.* 2009; Pini *et al.* 2010). In Lago della Costa *Larix* pollen and macrofossils [as well as pollen of other light demanding woody and herbaceous pioneers (*Juniperus, Betula, Artemisia,* Poaceae)] slightly increased in the period before the LGM. The authors suggest that steppic vegetation dominated the Po Plain whereas *Larix* colonized slopes around the lake. A similar landscape opening with occurrence of *Larix* and domination by xerophytes was documented in Lake Fimon. These results demonstrate that *Larix* was favoured during the Heinrich event 2 between 26.5 ka and 24.3 ka (Sánchez Goñi & Harrison 2010). Lake Fimon does not cover the LGM whereas in Lago della Costa pollen and macrofossils of *Larix* are also found during the period of the LGM between 23.5 ka and 19.5 ka (Mix *et al.* 2001). Hence, Lago della costa (Euganean Hills, Venetia) is identified as LGM refuge.

In the Pannonian Basin detailed inferences on the pre-LGM period are possible thanks to a recent investigation of Lake Fehér (86 m a.s.l.) in the Hungarian Plain (Sümegi *et al.* 2013). As in the two Alpine sites *Larix* was favoured by Heinrich event 2 during which forest declined and steppe expanded. Similar deforestation and desertification coinciding with

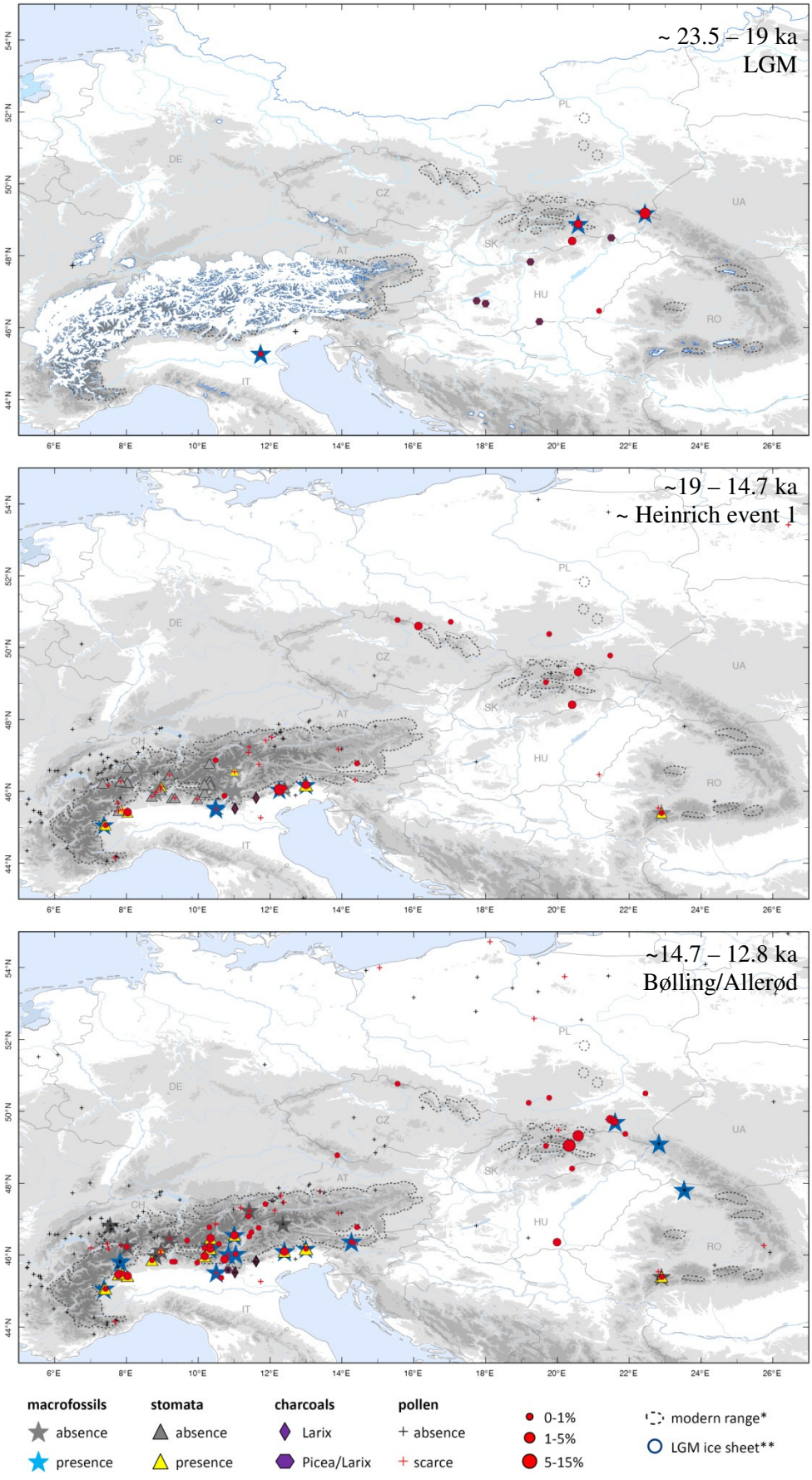

Figure 14 Fossil distribution of larch from 23.5 – 12.8 ka

Heinrich event 2 has also been observed in other European regions (e.g. Northwestern Iberia, Naughton *et al.* 2007), suggesting that Heinrich 2 event may also have affected *Larix* across its range. Moreover, studies in Lake Fehér also document two warm intervals leading to an increase of *Picea* and deciduous trees and a concomitant decrease of *Larix*. The authors assigned these warm intervals to D-O events 2 and 3 and corroborated the palynological evidence with malacological results (Sümegi *et al.* 2013). This study shows particularly well how millennial scale climate oscillations repeatedly impacted larch population in the early MIS 2.

During the LGM *Larix* can still be found in Lake Fehér and in addition in Nagymohos (394 m a.s.l.) located in the north-eastern Hungarian Mountains (Magyari 2002; Magyari *et al.* 1999). There are also charcoals from the Hungarian Plain dated to the LGM (Gaborí-Csánk 1960; Geyh *et al.* 1969; Vogel & Waterbolk 1964; Willis & van Andel 2004) identified as *Picea-Larix*. As both species were present in Lake Fehér this may indicate other *Larix* occurrences. In addition LGM persistence of larch is proven in the Western Carpathians by a continuous pollen curve and additional macrofossils from Šafárka as well as in the southeast Polish Bieszczady Mountains by a single probe with pollen and macrofossils from Smerek (600 m a.s.l.) dated back to ~20 ka (16925 ± 325 uncal. BP, Ralska-Jasiewiczowa 1980; Wacnik *et al.* 2004). There is also early pollen evidence dated ~20 ka from one site Labský důl (1039 m a.s.l.) in the Sudetes (PALYCZ; Engel *et al.* 2010; Jankovská 2004). However, the early part of the profile was poorly dated, and hence LGM persistence in the Sudetes cannot be proven solely on the basis of this record.

Post-LGM – onset of MIS 1

(19 - 14.7 ka, Mix *et al.* 2001; Svensson *et al.* 2008)

The period between the LGM and the onset of MIS 1 (~19 – 14.7 ka) can be studied based on a much larger number of sites compared to previous periods, including 88 with *Larix* evidence (Fig. 14, Table S 12, Supporting information). 35 of them document *Larix* occurrence since at least 18 ka. Fossils are abundant in the southern Alpine and Carpathian regions and are also found in a few sites of the Polish lowland as well as in the Pannonian basin. In the Alps regions of increased fossil abundance alternate with regions of reduced abundance in west-east direction, pointing to several localities were larch populations could persist (Turin region, Venetian region, South Carinthian region). Evidence preferentially

includes low altitudinal sites south to the Alps but scarce evidence was also found at some higher elevation sites and along the Adige-Inn valley, indicating first upward movements and Alpine transmigration starting from several places (Lake Garda region, Lake Como region, Turin region).

Fossil wood dated back to 18.3 ka is found in Lago Piccolo di Avigliana (353 m a.s.l), a finding embedded in a sediment layer above an even older but undated needle evidence. Together with conspicuous pollen and stomata evidence assigned to different phases of the Oldest Dryas (e.g. Lago di Viverone, 220 m a.s.l., Torfsee, 270 m a.s.l., Schneider 1978) this suggests that larch was already present in the lowlands when deglaciation started (Schneider 1978; Vescovi *et al.* 2007). Data suggest that the Turin region was the initial area of subsequent recolonization. Evidence from higher altitudes is very scarce and may indicate the presence of a few (krummholz-like) individuals near the site or the presence of trees at lower elevations.

There is a time lag between early deglaciation and pioneer forest establishment that has been dated back to ~ 15.8 – 16 ka (Vescovi *et al.* 2007). This may be explained by climatic constraints caused by Heinrich event 1 (~18 – 15.6 ka, Sánchez Goñi & Harrison 2010), which has been shown to have caused desertification in Western Iberia (Naughton *et al.* 2007). For this time treeline was estimated at ~400 – 500 m (Tinner 2007).

A first single but noteworthy pollen finding in the Maritime Alps was made in Selle di Carnino at ~16.2 ka (1905 m a.s.l.; ALPADABA; De Beaulieu 1977). It remains unclear whether it was wind-transported or of local origin. However, this raises the question of the origin of current larch populations from the Maritime Alps in France. Two hypotheses have been formulated (De Beaulieu 1977; Ortu 2002). First, they could originate from the Italian populations that survived in the Turin region. Second, the source populations could have persisted the LGM in the Maritime Alps, a region that was less glaciated than other parts of the Alps. De Beaulieu (1977) suggested for instance the French Mercantour as a possible refuge. A combination of both hypotheses can also be imagined.

In the regions of Lombardy and Ticino larch fossils are less abundant after the LGM which may be explained by the less continental conditions favouring other tree taxa (e.g. Lago di Biandronno, Suossa, Schneider 1978; Zoller & Kleiber 1971). Further east, in the Trentino-Adige and Venetian region, macrofossils and pollen findings are more abundant and indicate *Larix* growing in pioneer forests. Buried *Larix* trunks were found for instance in the Venetian

Prealps and dated back to 18.1 – 17 ka (Casadoro *et al.* 1976; Friedrich *et al.* 1999; Kromer *et al.* 1998). In addition, macrofossil, stomata and pollen records are documented in the Ragogna Lake located at the southern Alpine margin in northwestern Italy since ~ 17.1 ka (Monegato *et al.* 2007; Wick 1996). Evidence from the area around Lake Garda and scarce findings along the Adige-Inn valley suggest a migrational pathway across the Alps leading west to the Dolomites along a line from Lake Garda to Innsbruck through the Brenner pass, i.e. the lowest pass in this part of the Alps.

In the eastern Alpine margin (>14° E), fossil sites are scarce during this period. However, pollen evidence from ~18.7 ka exists in the Austrian region of Carinthia in the site Längsee (Schmidt *et al.* 1998). Given that maximum glacier extent during the LGM did not entirely cover the eastern Alpine margin and in view of the fact that many herbaceous endemics associated to upper montane coniferous forests grow there (Tribsch & Schönswetter 2003), it can be assumed that the Längsee record also points to a refuge of larch.

In the southern Carpathian region pollen and stomata are found in two high elevation sites in the Romanian Retezat mountains that are part of the Southern Carpathians (Lake Brazi, 1740 m a.s.l.; Lake Galeş, 1990 m a.s.l., Magyari *et al.* 2012). There is evidence for the presence of larch in both profiles since the onset of the record at ~15 ka. In the Retezat mountains maximum glacier advance was centred ~16-18 ka and the early high altitudinal larch occurrences seem to be best explained by refuges at lower elevations (Magyari *et al.* 2012). In the western Carpathians and their forelands as well as in the Sudetes larch fossils became abundant at this time suggesting that larch was more widespread in this region than today.

MIS 1 (since ~14.7 ka)

During MIS 1, which includes the late-glacial interstadial (~14.5 – 12.8 ka, Ammann *et al.* 2006; Litt *et al.* 2001; Rasmussen *et al.* 2006), the Younger Dryas (~12.8 – 11.7 ka, Ammann *et al.* 2006; Litt *et al.* 2001; Rasmussen *et al.* 2006) and the Holocene (since ~ 11.7 ka) *Larix* is observed in 283 fossil sites. The data show a great diversity of responses to climatic and anthropogenic changes depending on the geographical location, the topography of the mountain massifs and additional local factors. In the following the most important features of this history are described without going into the details from each individual site.

Late-glacial interstadial (Bølling/Allerød, 14.5 – 12.8 ka)

During the Bølling/Allerød (D-O 1) fossil abundance increases considerably indicating a conspicuously wide distribution range covering the Alps, the Sudetes, the Carpathians, the Pannonian Plain and the Polish lowland where the species is found far to the north (Fig. 14).

In the Alpine region the abrupt warming at the onset of the Bølling/Allerød triggered an altitudinal shift to higher elevations, documented by larch macrofossil, stomata and pollen records. Macrofossil occurrences above 1000 m a.s.l. were for instance found in the Pilaz peat bog (1900 m a.s.l.) in the Aosta Valley (Brugiapaglia 1997, 2007), the Totenmoos (1718 m a.s.l.) in the Adige Valley (Heiss *et al.* 2005) and the Palughetto mire (1040 m a.s.l.) in the Venetian Prealps (Avigliano *et al.* 2000). However, it has been shown that Alpine treelines with *Larix* and other treeline species remained about 600 m below the current level (Gobet *et al.* 2005; Tinner 2007; Tobolski & Ammann 2000) and that subalpine larch forests became established only after the Bølling/Allerød. At lower elevations *Larix* is still found, although other tree species became predominant (e.g. Finsinger *et al.* 2006; Monegato *et al.* 2007; Schneider 1978).

In the Carpathian region a detailed focus on altitudinal distribution is not possible due to the scarcity of sites >1000 m a.s.l. However, the two high altitudinal southern Carpathian sites and sites at 600 - 800 m a.s.l. in the northeastern and western Carpathians (Feurdean & Bennike 2004; Jankovská 1984, 1991; Magyari *et al.* 2012; Ralska-Jasiewiczowa 1980; Wohlfarth *et al.* 2001) demonstrate the presence of larch in the mountains and an increased abundance during this warming phase.

Younger Dryas (~12.8 – 11.7 ka)

The cold episode of the Younger Dryas (~12.8 – 11.7 ka, Fig. 15) led to heterogeneous responses of larch. This can be illustrated by the considerable variation of the upper forest line in the Alps (Gobet *et al.* 2005) and in the Carpathians. Larch responses ranged from a complete decline in some high altitudinal sites (e.g. Heiss *et al.* 2005; Jankovská 1984, 1991) to stability or even expansion at lower elevations (Andrič *et al.* 2009; Bortenschlager 1984; Koperowa 1962).

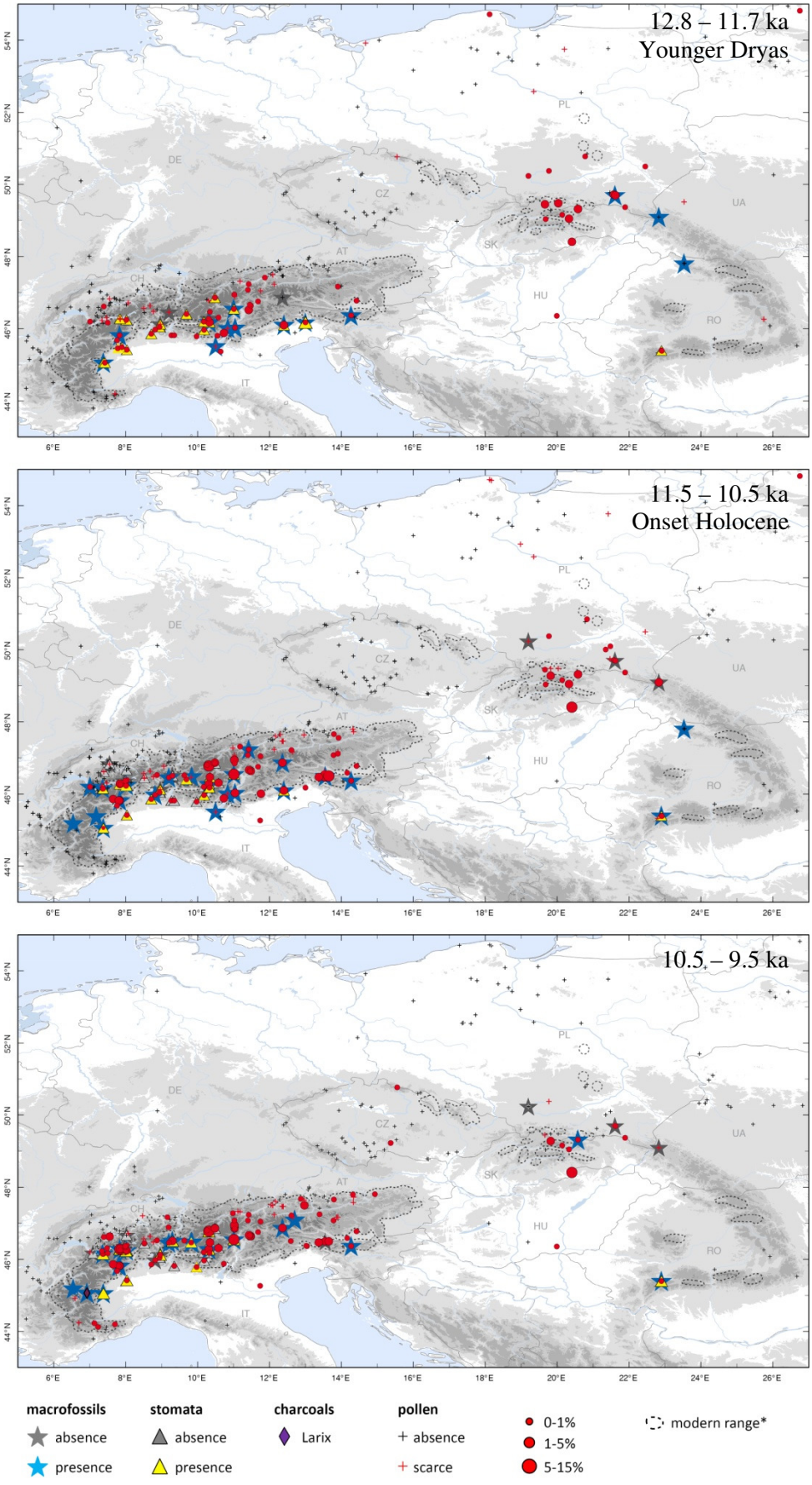

Figure 15 Fossil distribution of larch from 12.8 – 9.5 ka

Holocene (since ~11.7 ka)

The Holocene fossil distribution indicates maximum abundance during the first two millennia followed by successive decline at low altitudinal sites caused by the spread of competing tree species (Fig. 15 - 19). Modern species range restriction was reached at ~ 5ka.

At the beginning of the Holocene *Larix* rapidly moved upwards in response to an abrupt temperature rise of 4-6°C at ~11.5–11.4 ka causing a timberline shift of 800 m in 200-300 years (Tinner 2007). The establishment of larch subalpine forests has been studied very precisely and its timing has been estimated based on macrofossils from different high altitude sites in southern Switzerland and Northern Italy (Gobet *et al.* 2005). In the Valais and the Lombardy larch establishment has been dated around 11.4-11.0 ka whereas in Upper Engadine it happened 300 years later at ~10.7 ka. For the other parts of the Alps less data is available making it difficult to precisely estimate larch forest establishment. However, for the Western Alps macrofossils and a continuous pollen curve from a high altitudinal site in the Aosta valley point to subalpine forest establishment between 11 and 10.5 ka, which is in agreement with another pollen record from the same valley (Brugiapaglia 2007). In other parts of the Western Alps, subalpine larch forests appeared later. In the French Savoy, macrofossil data confirm the presence of larch since the onset of the Holocene, but forests got established only later at ~ 8 ka (Blarquez *et al.* 2009). For the Maritime Alps, no macrofossils were found and inferences are hampered by discontinuous pollen curves and differing signals among study sites (Ortu 2002), but there are indications that subalpine forests established at ~9.8 ka (Ortu *et al.* 2005). For the eastern Alpine region, data are also scarce but macrofossils from Eastern Tirol dated back to ~11.4 ka demonstrate the early arrival of the species (Oeggl & Wahlmüller 1994), even though subalpine forest development of the eastern parts of the Alps could have been delayed (Bortenschlager 1984; Gobet *et al.* 2005).

The combined record (macrofossils, stomata, pollen) from Lake Brazi (1740) in the southern Carpathians reveals that forest establishment was synchronous to that in the Central Alps (~11.2 ka). For other parts of Central Europe such precise correlations cannot be made due to the lack of adequate records. However, there is clear trend of upward shift of the treelines in the northwestern Carpathians, with decreasing pollen values at low elevation coinciding with increasing values at higher altitudinal sites (Koperowa 1962). In contrast, the distribution of larch in the Polish lowland north of the Carpathian area seems to have remained stable.

The first two millennia of the Holocene can hence be viewed as the key period to account for modern larch distribution. Moreover, during these two millennia larch dominated subalpine forests in the central Alps as it was favoured by high summer insolation and low winter insolation that limited the spread of *Pinus cembra* (Tinner & Kaltenrieder 2005). Treeline studies indicate that between 10 ka and 6 ka larch should have reached its uppermost position (Tinner 2007) and it has been shown that during this time larch forests were in dynamic equilibrium with climate including rapid changes such as the 8.2 cold event that led to the decline of larch populations in a site near the treeline (Tinner 2007; Tinner & Kaltenrieder 2005).

Since ~ 6 ka the timberline started to drop as a response to anthropogenically and climatically induced changes. While some human interventions led to the extinction of larch, others favoured the expansion of the species, which was observed in sites from across the range (e.g. Feurdean & Willis 2008; Kaltenrieder *et al.* 2005; Ortu *et al.* 2005; Schneider & Tobolski 1985). Prehistorical and historical epochs that had a major impact on larch were the Bronze Age, the Roman Age, the Middle Age and the Modern Age. During the Roman Age larch was favoured together with plants that are known to have long cultural histories. The species appears for instance in one site of the Maritime Alps during the Roman Age contemporaneously with chestnut (*Castanea*), walnut (*Juglans*), vine (*Vitis*) and cereals (Ortu *et al.* 2003), a coincidence that was also described in other regions (e.g. Schneider & Tobolski 1985). Land use changes, the role of fire and other anthropogenic disturbances favouring or limiting larch expansion are described in numerous publications. They will not be reviewed in this chapter, but it should be stressed that recent translocation history described in chapter 4 encompassing the last centuries has to be seen as one aspect of a 6 ka lasting anthropogenically shaped history during which multiple introductions and re-introductions of larch can be expected, even if they cannot be traced with paleontological tools. During the last 50 years larch history continued to be influenced by plantations and land abandonment in the Alpine region, suggesting that the species is currently expanding.

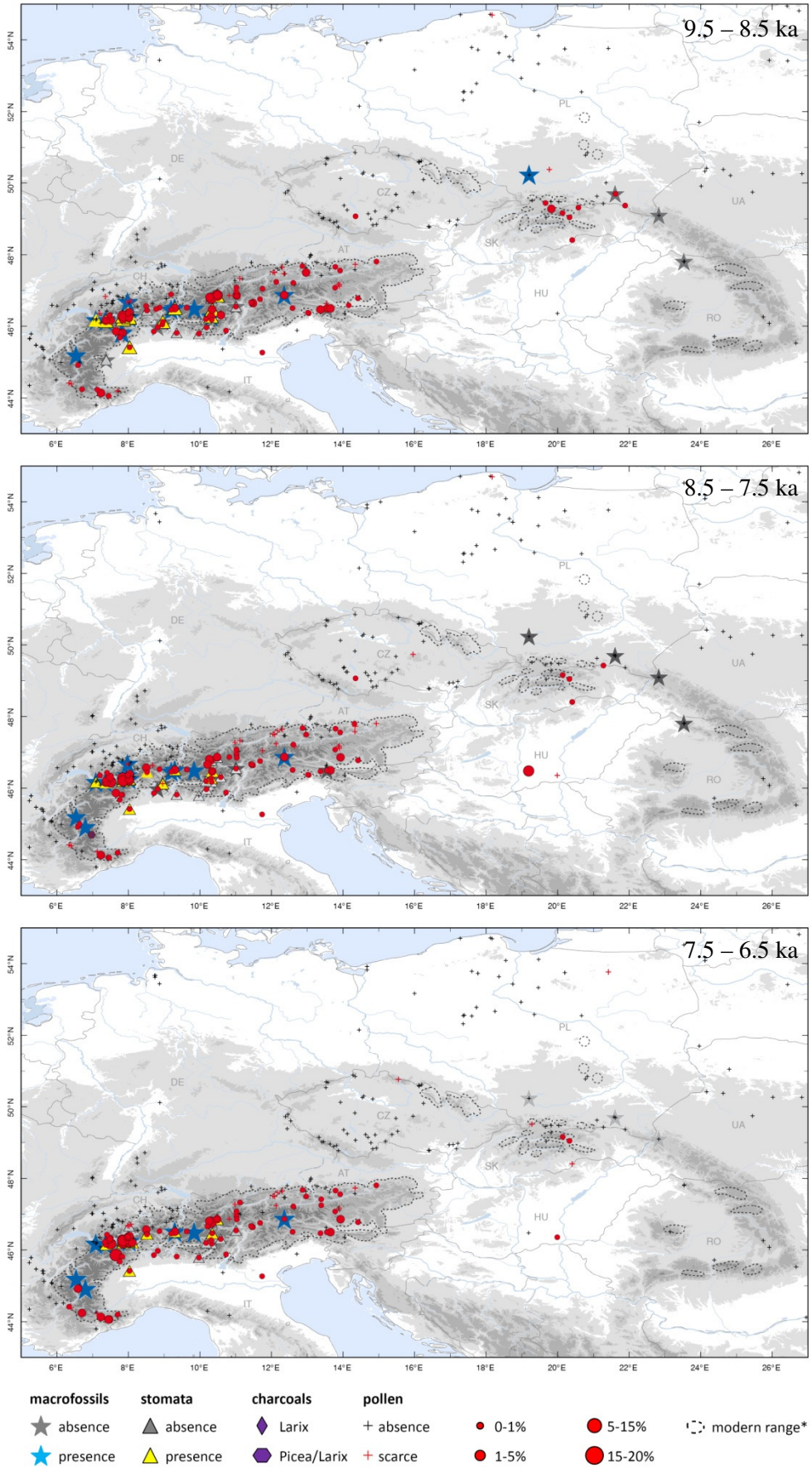

Figure 16 Fossil distribution of larch from 9.5 - 6.5 ka

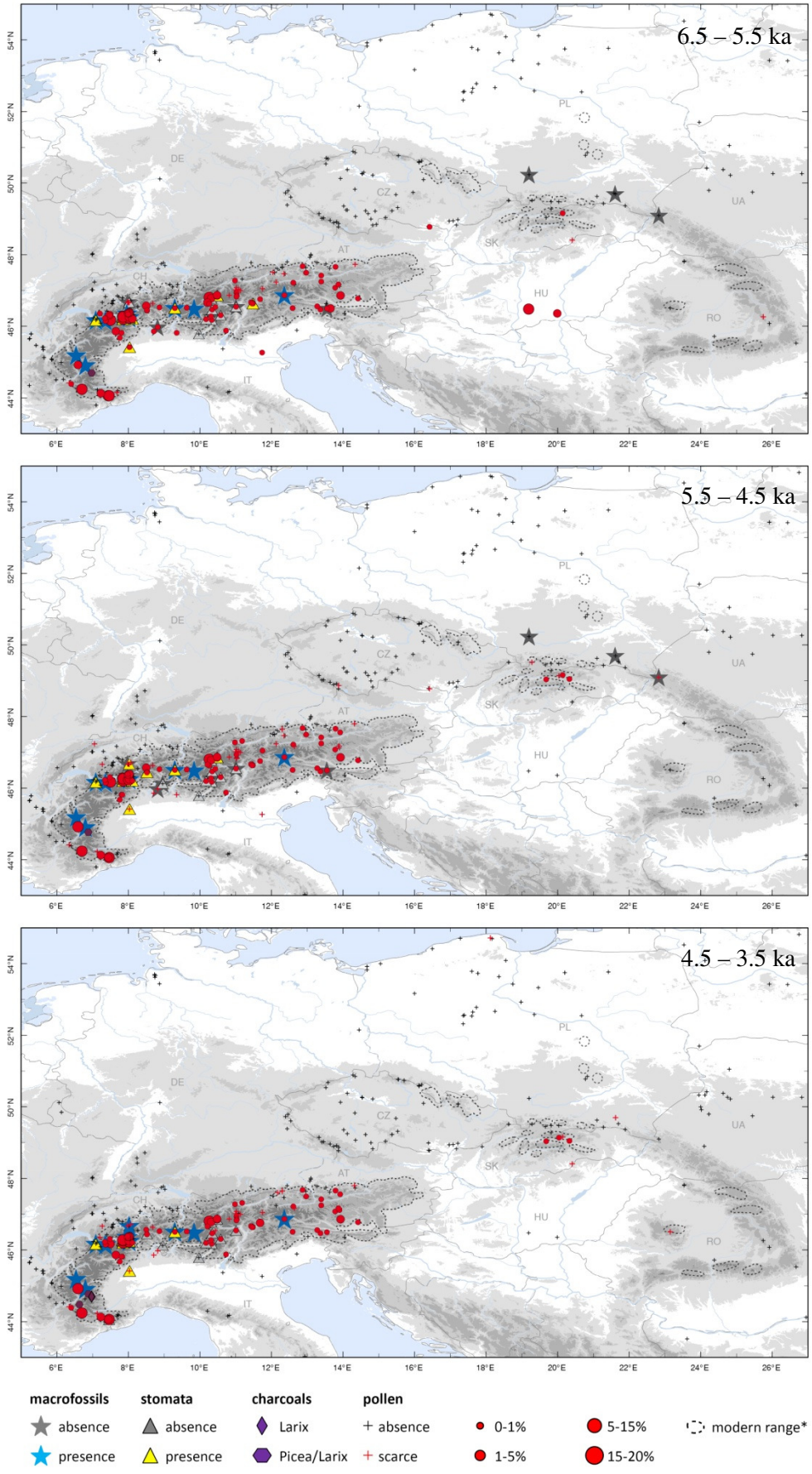

Figure 17 Fossil distribution of larch from 6.5 – 3.5 ka

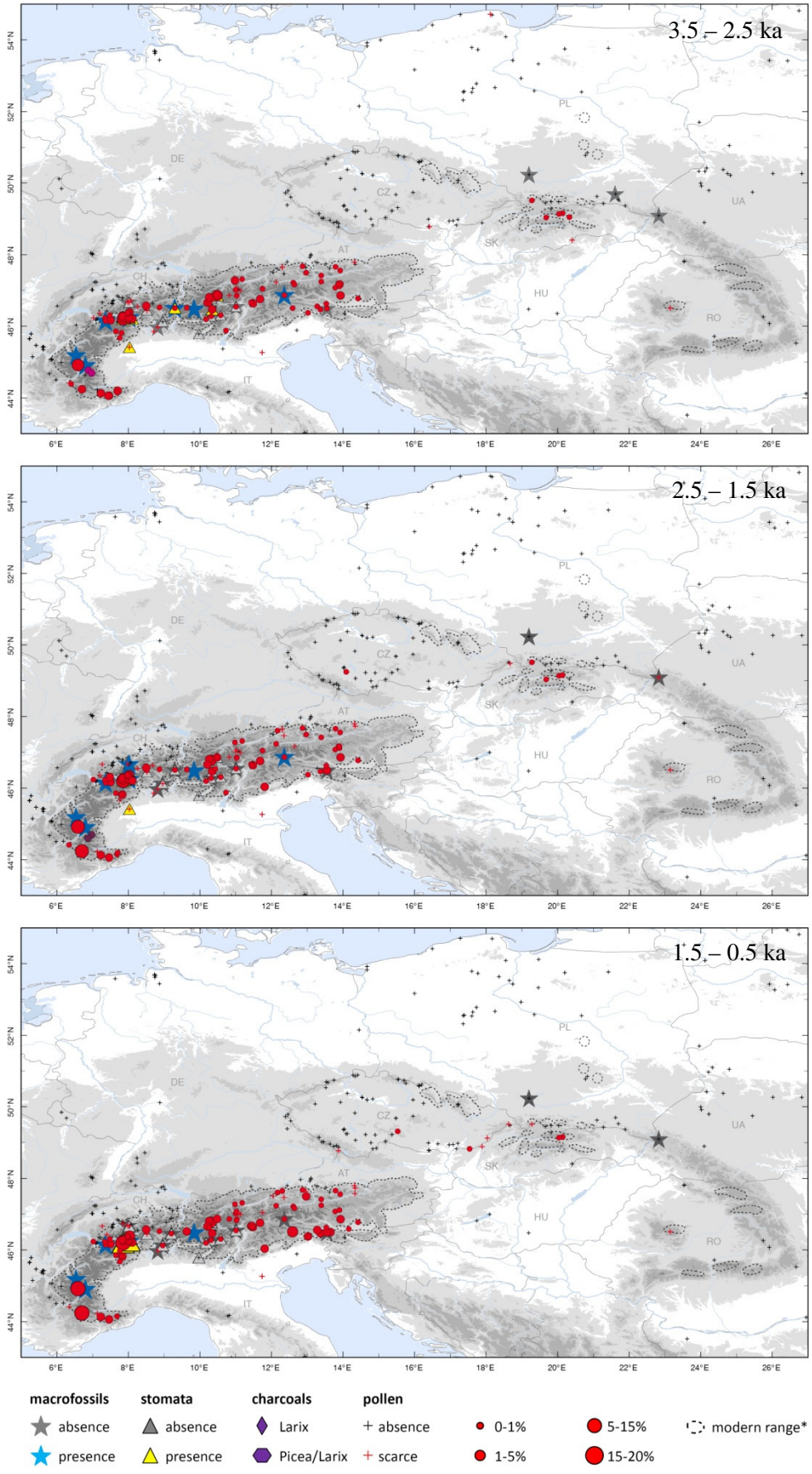

Figure 18 Fossil distribution of larch from 3.5 – 0.5 ka

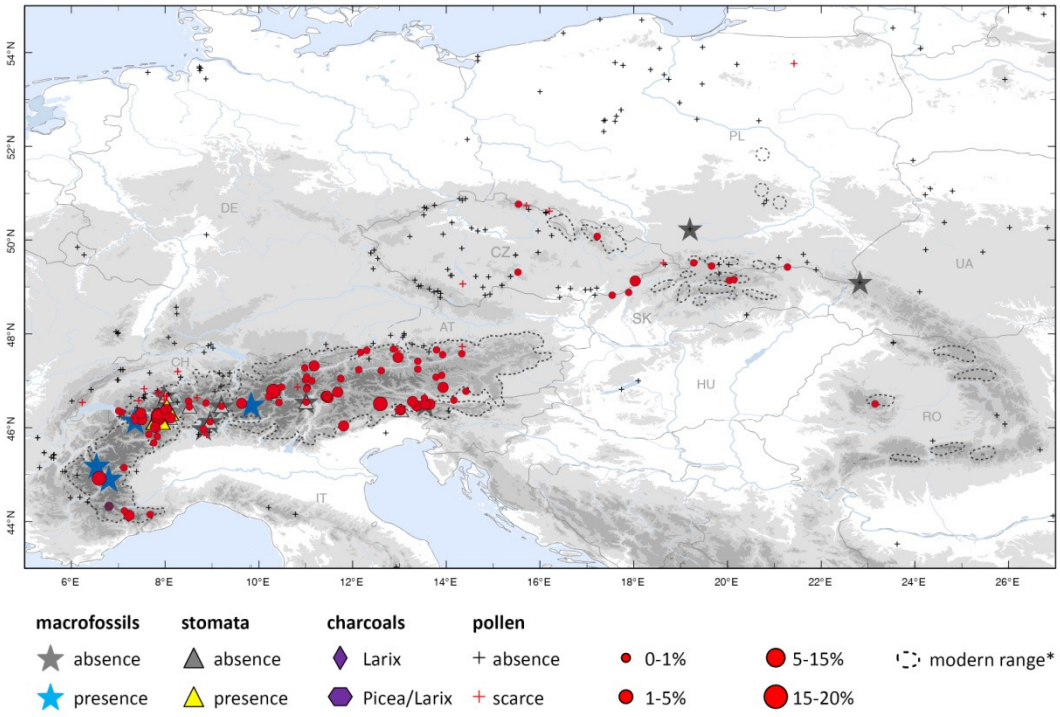

Figure 19 Fossil distribution of larch during the last 500 years

Summary and conclusions

MIS 5

- During the last interglacial *Larix* was restricted to high altitudinal sites. Climatic cooling at the transition to the first early Weichselian stadial favoured larch at lower elevations.
- The first early Weichselian stadial was dominated by tundra and steppe-like environments. Larch probably persisted at low elevations in the Alpine and Carpathian regions.
- The subcontinental climate of the first early Weichselian interstadial in north-central Europe has favoured the establishment of boreal forests. The intercalated Montaigu cold event triggered the rapid expansion of larch, leading to the largest distribution of the species up to now. This exceptionally broad distribution highlights the decisive impact that can have a rapid cold event superimposed to a sub-continental climate. In the Alpine region larch recolonized the mountain ranges and was favoured by the Montaigu event at lower elevations.

- The second Weichselian stadial was similar to the first and larch should have persisted at the same places.
- During the second early Weichselian interstadial larch expanded again in the north-central European lowlands and in the Alps. Compared to the first interstadial forest development was less pronounced. At the end of this interstadial forests finally declined in the north-central European lowlands.
- In the Alpine region there was a conspicuous advance of larch at mid-altitudes during the third early Weichselian interstadial.

MIS 4

- MIS 4 was one of the most severe climatic episode since the last 130 ka, causing major extinctions of larch populations and greatly restricting population sizes.

MIS 3

- During MIS 3 expansion of larch was favoured by D-O events 12 and 14 in the northern Alpine region. Distribution within this period was wide, covering the Alpine region, the Carpathian region, the Pannonian Plain and the Polish lowlands. Communities including larch were diverse (boreal forests, hemiboreal forests, forest tundra, forest steppes, tundra or steppes)

MIS 2

- In the pre-LGM part of MIS 2 the range of larch was very dynamic. The species was favoured by Heinrich event 2 as evidenced in the Alps and the Pannonian basin, whereas it was disfavoured by D-O 2 and 3 as seen in the Pannonian basin.
- During the LGM and after, larch persisted in the Alpine and the Carpathian foothills as well as in the Pannonian basin. The following areas can be considered as LGM-refuges: Turin region, Maritime Alps (?), Venetian region, southern Carinthia, Sudetes, western Carpathians (Tatra Mts) and southern Carpathians.
- During early deglaciation larch recolonized rapidly territories recently freed from the ice and built light pioneer forest at low elevations. Major forest development seemed to have been constrained by Heinrich event 1 (which was cooler than the LGM in western Europe)

MIS 1

- The Bølling/Allerød interstadial (D-O 1) triggered a rapid spread of larch to higher elevations and across the Polish plain leading to a wide distribution range. However, the tree line did not reach the level observed in the Holocene.
- The Younger Dryas caused heterogeneous responses ranging from clear decline at high elevations, no significant change or expansion at low elevation.
- The rapid temperature rise at the beginning of the Holocene triggered a rapid colonization of high elevation mountain ranges. Subalpine larch forests became established during the first two millennia of the Holocene and appeared to be in dynamic equilibrium with climate. Low elevation populations were successively replaced by other species. The species decreased until its modern distribution range was reached at ~ 5 ka.
- Anthropogenic impacts on larch populations became important at the Bronze Age and remained high until Modern Times. Human impacts were varied, ranging from extinctions close to the treeline and increased abundance and colonization of new areas, which led to disequilibrium between larch populations and climate. Recent land abandonment by agriculture should result in continuing larch expansion.

Abrupt climate events that (may) have influenced the history of larch

- Montaigu event
- D-O events 1, 2, 3, 12, 14
- Heinrich events 1, 2, 6 (?)
- 8.2 event
- Onset of Younger Dryas
- Onset of Holocene

This account of the history of larch since the last interglacial shows that the species has persisted during the last 130,000 years close to its modern distribution range while at the same time reacting rapidly to short episodes of climate change. This illustrates the flexibility of larch towards abrupt climate change in the long-term. In the short-term, anthropogenic changes resulted in disequilibrium between larch distribution and climate, with new habitats artificially created quickly occupied by the species and artificial admixture of sometimes distant and divergent populations (see chapter 4). This specificity should be taken into account in future attempts to estimate how the species will respond to forecasted temperature increase.

Supporting Information

Table S 6 Fossil sites from 130 – 112 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Reference
Amersfoort 3	52.16	5.39	<100	NL	X	0	Zagwijn (1961)
Aschenhütte	51.68	10.30	240	DE	0	0	Ricken & Grüger (1988)
Azzano Decimo	45.89	12.71	10	IT	X	0	Pini *et al.* (2009)
Barendorf 2 and 3	53.23	10.52	74	DE	X	0	Freund *et al.* (1997)
Brørup	55.48	9.02	<100	DK	0	0	Andersen (1961)
Dziernakowo	53.10	23.62	164	PL	X	0	Kupryjanowicz (2008)
Füramoos	47.98	9.88	662	DE	X	0	Müller (2000, 2003)
Gondiswil	47.15	7.87	640	CH	0	1	Wegmüller (1992)
Gröbern	51.69	12.46	107	DE	0	0	Litt (1990, 1994), Mai (1990)
Groß Todtshorn	53.21	9.78	<100	DE	0	0	Caspers (1997), Freund (1997a)
Horoszki Duze	52.26	23.01	180	PL	1	1	Granoszewski (2003
Ioannina	39.75	20.85	470	GR	X	0	Tzedakis (2000), Tzedakis *et al.* (2002)
Jammertal	48.10	9.73	578	DE	X	1	Müller (2001, 2000)
Keller	54.08	9.43	<100	DE	X	0	Menke (1970)
Kittlitz	51.84	13.93	170	DE	X	1	Erd (1973)
La Grande Pile	47.73	6.50	330	FR	X	0	Woillard (1975, 1978), de Beaulieu & Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	Reille & de Beaulieu (1990)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	Allen *et al.* (1999)
Lake Fimon	45.47	11.53	23	IT	X	1	Pini *et al.* (2010)
Les Echets	45.87	4.92	267	FR	X	0	de Beaulieu & Reille (1984, 1989)
Lunteren	52.09	5.62	<100	NL	X	0	Zagwijn (1961)
Margreteberg	56.77	12.74	<100	SE	X	1	Påsse *et al.* (1988)
MD04-2845	45.35	-5.22	-4100	NA	X	0	Sánchez Goñi *et al.* (2008)
MD95-2042	37.80	-10.17	-3148	NA	X	0	Sánchez Goñi *et al.* (1999)
Mondsee	47.85	13.35	540	AT	0	1	Klaus (1975), Drescher-Schneider (2000), Oeggl & Unterfrauner (2000)
Neheim-Hüsten	51.45	7.97	<100	DE	N	0	Teunissen *et al.* (1972)
Neuenhausen-Veldhausen	52.52	6.99	<100	DE	X	0	Freund (1997b)
Odderade 5	54.14	9.19	<100	DE	0	0	Averdieck (1967)
Oerel	53.48	9.06	<100	DE	0	0	Behre & Lade (1986), Behre *et al.* (2005)
Osterwanna I and II	53.74	8.80	<100	DE	X	0	Behre (1974)
Quakenbrück	52.67	7.96	<100	DE	0	0	Hahne *et al.* (1994a)
Rederstall I and II	54.23	9.21	<100	DE	X	1	Menke & Tynni (1984)
Ribains	44.84	3.82	1192	FR	X	0	de Beaulieu & Reille (1992b)
Samerberg 1973	47.75	12.20	612	DE	1	1	Grüger (1979)
Sokli	67.08	29.18	220	FI	X	1	Helmens *et al.* (2000, 2007), Engels *et al.* (2010)
Solniki	53.50	23.20	143	PL	X	1	Kupryjanowicz (2008)
Stenberget	55.52	13.55	<100	SE	X	1	Berglund & Lagerlund (1981)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	Wijmstra (1969)
Valle di Castiglione	41.88	12.76	44	IT	X	0	Follieri *et al.* (1988, 1998)
Wladyslawow	52.13	18.47	<100	PL	0	1	Tobolski (1986, 1991)
Zgierz-Rudunki	51.84	19.42	200	PL	0	1	Jastzebska-Mamelka (1985)

*1: presence, 0: absence, X: not investigated; M: Macrofossils; P: Pollen

Table S 7 Fossil sites from 109 – 87 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Reference
Amersfoort 3	52.16	5.39	<100	NL	X	0	Zagwijn (1961)
Aschenhütte	51.68	10.30	240	DE	0	1	Ricken & Grüger (1988)
Azzano Decimo	45.89	12.71	10	IT	X	0	Pini *et al.* (2009)
Barendorf 2 and 3	53.23	10.52	74	DE	X	1	Freund *et al.* (1997)
Brørup	55.48	9.02	<100	DK	0	1	Andersen (1961)
Dziernakowo	53.10	23.62	164	PL	X	1	Kupryjanowicz (2008)
Füramoos	47.98	9.88	662	DE	X	1	Müller (2000, 2003)
Gondiswil	47.15	7.87	640	CH	0	1	Wegmüller (1992)
Gröbern	51.69	12.46	107	DE	0	1	Litt (1990, 1994), Mai (1990a)
Groß Todtshorn	53.21	9.78	NA	DE	0	1	Caspers (1997), Freund (1997)
Horoszki Duze	52.26	23.01	180	PL	1	1	Granoszewski (2003)
Hunteburg	52.44	8.28	<100	DE	0	1	Hahne (1994b)
Ioannina	39.75	20.85	470	GR	X	0	Tzedakis (2000), Tzedakis et al. (2002)
Jammertal	48.10	9.73	578	DE	X	1	Müller (2000, 2001)
Keller	54.08	9.43	<100	DE	X	1	Menke (1970)
Kittlitz	51.84	13.93	170	DE	X	1	Erd (1973)
La Grande Pile	47.73	6.50	330	FR	X	1	Woillard (1975, 1978), de Beaulieu & Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	Reille & de Beaulieu (1990)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	Allen *et al.* (1999)
Lake Fimon	45.47	11.53	23	IT	X	1	Pini *et al.* (2010)
Les Echets	45.87	4.92	267	FR	X	0	de Beaulieu & Reille (1984, 1989)
Lunteren	52.09	5.62	<100	NL	X	0	Zagwijn (1961)
MD04-2845	45.35	-5.22	-4100	NA	X	0	Sánchez Goñi *et al.* (2008)
MD95-2042	37.80	-10.17	-3148	NA	X	0	Sánchez Goñi *et al.* (1999)
Mondsee	47.85	13.35	540	AT	0	1	Klaus (1975), Drescher-Schneider (2000), Oeggl & Unterfrauner (2000)
Neheim-Hüsten	51.45	7.97	<100	DE	0	0	Teunissen *et al.* (1972)
Neuenhausen-Veldhausen	52.52	6.99	<100	DE	X	1	Freund (1997b)
Odderade 5	54.14	9.19	<100	DE	0	1	Averdieck (1967)
Oerel	53.48	9.06	<100	DE	0	1	Behre & Lade (1986), Behre *et al.* (2005)
Osterwanna I and II	53.74	8.80	<100	DE	X	1	Behre (1974)
Padul	37.00	-3.07	785	ES	X	0	Pons & Reille (1988)
Quakenbrück	52.67	7.96	<100	DE	0	1	Hahne *et al.* (1994a)
Rederstall I and II	54.23	9.21	<100	DE	X	1	Menke & Tynni (1984)
Ribains	44.84	3.82	1192	FR	X	0	de Beaulieu & Reille (1992b)
Samerberg 1973	47.75	12.20	612	DE	1	1	Grüger (1979)
Sokli	67.08	29.18	220	FI	X	1	Helmens *et al.* (2000, 2007), Engels *et al.* (2010)
Solniki	53.50	23.20	143	PL	X	1	Kupryjanowicz (2008)
Stenberget	55.52	13.55	<100	SE	X	1	Berglund & Lagerlund (1981)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	Wijmstra (1969)
Valle di Castiglione	41.88	12.76	44	IT	X	0	Follieri *et al.* (1988, 1998)
Wladyslawow	52.13	18.47	<100	PL	0	1	Tobolski (1986, 1991)
Zgierz-Rudunki	51.84	19.42	200	PL	0	1	Jastzebska-Mamelka (1985)

***1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen**

Table S 8 Fossil from 83 – 78.2 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Reference
Azzano Decimo	45.89	12.71	10	IT	X	0	Pini *et al.* (2009)
Dziernakowo	53.10	23.62	164	PL	X	1	Kupryjanowicz (2008)
Füramoos	47.98	9.88	662	DE	X	1	Müller (2000, 2003)
Gondiswil	47.15	7.87	640	CH	0	1	Wegmüller (1992)
Gröbern	51.69	12.46	107	DE	1	1	Litt (1990, 1994), Mai (1990)
Groß Todtshorn	53.21	9.78	NA	DE	0	1	Caspers (1997), Freund (1997a)
Horoszki Duze	52.26	23.01	180	PL	1	1	Granoszewski (2003)
Hunteburg	52.44	8.28	<100	DE	0	1	Hahne *et al.* (1994b)
Ioannina	39.75	20.85	470	GR	X	0	Tzedakis (2000), Tzedakis *et al.* (2002)
Jammertal	48.10	9.73	578	DE	X	1	Müller (2000, 2001)
Kittlitz	51.84	13.93	170	DE	X	1	Erd (1973)
La Grande Pile	47.73	6.50	330	FR	X	1	Woillard (1975, 1978), de Beaulieu & Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	Reille & de Beaulieu (1990)
Lagaccione	42.57	42.57	355	IT	X	0	Follieri *et al.* (1998), Magri (1999)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	Allen *et al.* (1999)
Lake Fimon	45.47	11.53	23	IT	X	1	Pini *et al.* (2010)
Les Echets	45.87	4.92	267	FR	X	1	de Beaulieu & Reille (1984, 1989)
MD04-2845	45.35	-5.22	-4100	-	X	0	Sánchez Goñi *et al.* (2008)
MD95-2042	37.80	-10.17	-3148	-	X	0	Sánchez Goñi *et al.* (1999)
Mondsee	47.85	13.35	540	AT	1	1	Klaus (1975), Drescher-Schneider (2000), Oeggl & Unterfrauner (2000)
Neheim-Hüsten	51.45	7.97	<100	DE	0	0	Teunissen *et al.* (1972)
Odderade 5	54.14	9.19	<100	DE	0	1	Averdieck (1967)
ODP site 976	36.20	-4.30	-1108		X	0	Combourieu-Nebout *et al.* (2002)
Oerel	53.48	9.06	<100	DE	1	1	Behre and Lade (1986), Behre *et al.* (2005)
Padul	37.00	-3.07	785	ES	X	0	Pons & Reille (1988)
Quakenbrück	52.67	7.96	<100	DE	0	1	Hahne *et al.* (1994a)
Rederstall I and II	54.23	9.21	<100	DE	X	1	Menke & Tynni (1984)
Ribains	44.84	3.82	1192	FR	X	0	de Beaulieu & Reille (1992b)
Samerberg 1973	47.75	12.20	612	DE	1	1	Grüger (1979)
Sokli	67.08	29.18	220	FIN	X	0	Helmens *et al.* (2000, 2007), Engels *et al.* (2010)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	Wijmstra (1969)
Valle di Castiglione	41.88	12.76	44	IT	X	0	Follieri *et al.* (1988, 1998)
Wladyslawow	52.13	18.47	<100	PL	0	1	Tobolski (1986, 1991)
Zgierz-Rudunki	51.84	19.42	200	PL	1	1	Jastzebska-Mamelka (1985)

*1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen

Table S 9 Fossil sites from 73.5 – 59.4 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Reference
Abric Romaní	41.53	1.68	350	-	X	0	Burjachs & Julià (1994)
Azzano Decimo	45.89	12.71	10	IT	X	0	Pini *et al.* (2009)
Füramoos	47.98	9.88	662	DE	X	1	Müller (2000, 2003)
Gondiswil	47.15	7.87	640	CH	0	1	Wegmüller (1992)
Ioannina	39.75	20.85	470	GR	X	0	Tzedakis (2000), Tzedakis *et al.* (2002)
La Grande Pile	47.73	6.50	330	F	X	0	Woillard (1975, 1978), de Beaulieu & Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	F	X	0	Reille & de Beaulieu (1990)
Lagaccione	42.57	42.57	355	IT	X	0	Follieri *et al.* (1998), Magri (1999)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	Allen *et al.* (1999)
Lake Fimon	45.47	11.53	23	IT	X	1	Pini *et al.* (2010)
Les Echets	45.87	4.92	267	F	X	1	de Beaulieu & Reille (1984, 1989)
MD04-2845	45.35	-5.22	-4100	X	X	0	Sánchez Goñi *et al.* (2008)
MD95-2039	40.58	-10.35	-3381	X	X	0	Roucoux *et al.* (2001)
MD95-2042	37.80	-10.17	-3148	X	X	0	Sánchez Goñi *et al.* (1999)
Megali Limni	39.10	26.32	323	X	X	0	Margari *et al.* (2007, 2009)
ODP site 976	36.20	-4.30	-1108	-	X	0	Combourieu-Nebout *et al.* (2002)
Oerel	53.48	9.06	<100	DE	0	0	Behre & Lade (1986), Behre *et al.* (2005)
Padul	37.00	-3.07	785	ES	X	0	Pons & Reille (1988)
Polanów Samborzecki	50.63	21.67	165	PL	X	1	Komar *et al.* (2009)
Ribains	44.84	3.82	1192	F	X	0	de Beaulieu & Reille (1992b)
Samerberg 1973	47.75	12.20	612	DE	0	0	Grüger (1979)
Sokli	67.08	29.18	220	FI	X	0	Helmens *et al.* (2000, 2007), Engels *et al.* (2010)
Stracciacappa	42.13	12.32	220	X	X	0	Giardini (2007)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	Wijmstra (1969)
Valle di Castiglione	41.88	12.76	44	IT	X	0	Follieri *et al.* (1988, 1998)

*1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen

Table S 10 Fossil sites from 59.4 – 27.8 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Ch*	Reference
Abric Romaní	41.53	1.68	350	-	X	0	X	Burjachs & Julià (1994)
Azzano Decimo	45.89	12.71	10	IT	X	0	X	Pini *et al.* (2009)
Baggagera	45.74	9.39	260	IT	X	0	1	Maspero (1996)
Bialy Kościól	50.72	17.03	180	PL	1	1	X	Komar *et al.* (2009)
Bohunice	49.17	16.58	<100	CZ	X	X	1	Svoboda & Svoboda (1985), Damblon *et al.* (1996), Musil (2003), Willis & van Andel (2004)
Brzeźnica A	50.10	21.48	130	PL	1	1	X	Mamakowa & Starkel (1974)
Brzeźnica B	50.10	21.48	130	PL	1	1	X	Mamakowa & Starkel (1974)
Bulhary	48.83	16.75	166	CZ	1	1	X	Rybnikova & Rybnicek (1991)
Erd	47.48	18.91	200	HU	X	X	1	Musil (2003)
Füramoos	47.98	9.88	662	DE	X	1	X	Müller (2000, 2003)
Ioannina	39.75	20.85	470	GR	X	0	X	Tzedakis (2000), Tzedakis *et al.* (2002)
Istálloskö	48.16	20.42	330	HU	X	X	1	Musil (2003)
Jablůnka	49.38	17.95	350	CZ	X	1	X	Jankovska (2003), Jankovská & Pokroný (2008)
Kulna	49.51	16.61	300	CZ	X	X	1	Musil (2003)
La Grande Pile	47.73	6.50	330	FR	X	0	X	Woillard (1975, 1978), de Beaulieu & Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	X	Reille & de Beaulieu (1990)
Lagaccione	42.57	42.57	355	IT	X	0	X	Follieri *et al.* (1998), Magri (1999)
Lago della costa	45.27	11.74	7	IT	1	1	X	Kaltenrieder *et al.* (2009)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	X	Allen *et al.* (1999)
Lake Banyoles	42.13	2.75	173	-	X	0	X	Pérez-Obiol & Julia (1994)
Lake Fimon	45.47	11.53	23	IT	X	1	X	Pini *et al.* (2010)
Lake Xinias	39.05	22.27	500	-	X	0	X	Bottema (1979)
Les Echets	45.87	4.92	267	FR	X	1	X	de Beaulieu & Reille (1984, 1989)
MD04-2845	45.35	-5.22	-4100	-	X	0	X	Sánchez Goñi *et al.* (2008)
MD95-2039	40.58	-10.35	-3381	-	X	0	X	Roucoux *et al.* (2001)
MD95-2042	37.80	-10.17	-3148	-	X	0	X	Sánchez Goñi *et al.* (1999)
MD95-2043	36.14	-2.62	-1841	-	X	0	X	Sánchez Goñi *et al.* (2002)
Megali Limni	39.10	26.32	323	-	X	0	X	Margari *et al.* (2007, 2009)
Navarrés	39.10	-0.68	225	-	X	0	X	Carrión & Van Geel (1999)
ODP site 976	36.20	-4.30	-1108	-	X	0	X	Combourieu-Nebout *et al.* (2002)
Padul	37.00	-3.07	785	ES	X	0	X	Pons & Reille (1988)
Pavlov	48.87	16.67	270	CZ	X	1	1	Opravil (1994), Damblon *et al.* (1996), Damblon (1997)
Polanów Samborzecki	50.63	21.67	165	PL	X	1	X	Komar *et al.* (2009)
Prague-Podbaba	50.11	14.39	190	CZ	1	1	X	Jankovská & Pokroný (2008)
Ribains	44.84	3.82	1192	FR	X	0	X	de Beaulieu & Reille (1992b)
Riparo Fumane	45.56	10.92	350	IT	X	0	1	Maspero (1996)
Šafárka	48.88	20.58	600	SK	1	1	X	Jankovská *et al.* (2002), Jankovská & Pokroný (2008)

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Ch*	Reference
Sokli	67.08	29.18	220	FI	X	0	X	Helmens *et al.* (2000, 2007), Engels *et al.* (2010)
Spadzista Street	50.05	19.92	<100	PL	X	X	1	Musil (2003)
Stracciacappa	42.13	12.32	220	-	X	0	X	Giardini (2007)
Stránska skàlà	49.18	16.67	<100	CZ	X	X	1	Musil (2003), Damblon *et al.* (1996), Willis & van Andel (2004)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	X	Wijmstra (1969)
Týn n. Bečvou	49.51	17.62	370	CZ	X	1	X	Jankovská pers. comm.
Valle di Castiglione	41.88	12.76	44	IT	X	0	X	Follieri *et al.* (1988, 1998)
Věstonice I	48.87	16.63	255	CZ	X	X	1	Opravil (1994), Damblon *et al.* (1996), Damblon (1997), Slaviková-Veslá (1950), Willis& van Andel (2004)
Věstonice II	48.88	16.63	255	CZ	X	X	1	Mason *et al.* (1994), Opravil (1994), Damblon *et al.* (1996), Damblon (1997), Musil (2003), Willis & van Andel (2004)
Voka	59.41	27.60	22	EE	X	1	X	Bolikhovskaya & Molodkov (2007)
Willendorf	48.32	15.40	220	AU	X	X	1	Haesaerts *et al.* (1996), Damblon & Haesaerts (1997), Damblon (1997), Willis& van Andel (2004)
-	47.17	18.00	<100	HU	X	X	1	Geyh *et al.* (1969), Willis & van Andel (2004)
-	48.33	21.25	200	HU	X	X	1	Krolopp (1977), Willis & van Andel (2004)

*1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen, Ch: Charcoals

Table S 11 Fossil sites from 27.8 – 19 ka reported in the literature

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Ch*	St*	Reference
Azzano Decimo	45.89	12.71	10	IT	X	0	X	X	Pini *et al.* (2009)
Fehér-tó	46.47	21.16	86	HU	X	1	X	X	Magyari (2002)
Ioannina	39.75	20.85	470	GR	X	0	X	X	Tzedakis (2000) Tzedakis *et al.* (2002)
La Grande Pile	47.73	6.50	330	FR	X	0	X	X	Woillard (1975, 1978), de Beaulieu& Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	X	X	Reille& de Beaulieu (1990)
Lagaccione	42.57	42.57	355	IT	X	0	X	X	Follieri *et al.* (1998), Magri (1999)
Lago della costa	45.27	11.74	7	IT	1	1	X	X	Kaltenrieder *et al.* (2009)
Lago di Origlio	46.05	8.94	416	IT	X	0	X	0	Vescovi *et al.* (2007), Tinner *et al.* (1999)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	X	X	Allen *et al.* (1999)
Lago Piccolo di Avigliana	45.06	7.38	353	IT	Si	X	X	0	Finsinger *et al.* (2006), Finsinger& Tinner (2006), Vescovi *et al.* (2007)
Lake Banyoles	42.13	2.75	173	-	X	0	X	X	Pérez-Obiol& Julia (1994)
Lake Fimon	45.47	11.53	23	IT	X	1	X	X	Pini *et al.* (2010)
Lake Xinias	39.05	22.27	500	-	X	0	X	X	Bottema (1979)
Längsee	46.78	14.42	548	AT	X	0	X	X	Schmidt (1998)
MD95-2039	40.58	-10.35	-3381	-	X	0	X	X	Roucoux *et al.* (2001)
MD95-2042	37.80	-10.17	-3148	-	X	0	X	X	Sánchez Goñi *et al.* (1999)
MD95-2043	36.14	-2.62	-1841	-	X	0	X	X	Sánchez Goñi *et al.* (2002)
MD99-2331	41.15	-9.68	-2110	-	X	0	X	X	Naughton *et al.* (2007)
Megali Limni	39.10	26.32	323	-	X	0	X	X	Margari *et al.* (2007, 2009)
Nagymohos	48.41	20.41	394	HU	X	1	X	X	Magyari *et al.* (1999), Magyari (2002)
Navarrés	39.10	-0.68	225	-	X	0	X	X	Carrión& Van Geel (1999)
ODP site 976	36.20	-4.30	-1108	-	X	0	X	X	Combourieu-Nebout *et al.* (2002)
Padul	37.00	-3.07	795	-	X	0	X	X	Pons& Reille (1988)
Podgrodzie	50.01	21.35	-	PL	1	1	X	X	Mamakowa& Starkel (1977)
Ribains	44.84	3.82	1192	FR	X	0	X	X	de Beaulieu& Reille (1992b)
Šafárka	48.88	20.58	600	SK	1	1	X	X	Jankovská *et al.* (2002), Jankovská& Pokroný (2008)
Smerek	49.18	22.44	600	PL	1	1	X	X	Ralska-Jasiewiczowa (1980), Wacnik *et al.*(2004)
Spadzista Street	50.05	19.92	-	PL	X	X	1	X	Damblon *et al.* (1996), Musil (2003), Willis and van Andel (2004)
Stracciacappa	42.13	12.32	220	-	X	0	X	X	Giardini (2007)
Tenagi Philippon II	41.02	24.03	40	GR	X	0	X	X	Wijmstra (1969)
Valle di Castiglione	41.88	12.76	44	IT	X	0	X	X	Follieri *et al.* (1988, 1998)
Věstonice I	48.87	16.63	-	CZ	X	X	1	X	Opravil (1994), Damblon *et al.* (1996), Damblon (1997), Slaviková-Veslá (1950), Willis& van Andel (2004)
-	47.83	19.25	250	HU	X	X	1	X	Geyh *et al.* (1969), Willis& van Andel (2004)
-	46.67	18.00	200	HU	X	X	1	X	Gabori-Csánk (1960), Willis& van Andel (2004)

Site	Latdd	Londd	Alt m asl	Country	M*	P*	Ch*	St*	Reference
-	46.17	19.50	-	HU	X	X	1	X	Dobosi (1967); Willis& van Andel (2004)
-	48.50	21.50	-	HU	X	X	1	X	Vértes (1964), Willis& van Andel (2004)
-	46.75	17.75	50	HU	X	X	1	X	Vogel-Waterbolk (1964), Willis& van Andel (2004)
-	46.92	18.17	-	HU	X	X	1	X	Krolopp (1977), Marosi& Szilárd (1974); Willis& van Andel (2004)
-	47.75	21.25	-	HU	X	X	1	X	Geyh *et al.* (1969); Willis& van Andel (2004)
-	47.83	18.83	-	HU	X	X	1	X	Pécsi (1977), Willis& van Andel (2004)

*1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen, Ch: Charcoals, St: Stomata

Table S 12 Fossil sites from 19 – 14.7 ka reported in the literature

Site	Latdd	Londd	Alt m a.s.l.	Country	M*	P*	Ch*	St*	Reference
Azzano Decimo	45.89	12.71	10	IT	X	0	X	X	Pini *et al.* (2009)
Ballmoos	47.54	9.49	943	CH	X	0	X	X	Kral (1979)
Bialy Kościól	50.72	17.03	180	PL	X	1	X	X	Komar *et al.* (2009)
Bondone	46.03	11.04	1550	IT	0	0	X	X	Grüger (1968)
Bondone	45.81	10.55	1550	IT	X	0	X	X	Grüger (1968), Kral (1979)
Buchensee	47.77	8.98	430	DE	X	0	X	X	Bertsch (1961), Kral (1979)
Campra	46.53	8.87	1420	CH	X	0	X	X	Müller (1972)
Col di Val Bighera	46.28	10.31	2087	IT	0	0	X	0	Gehring (1997)
Etrach	47.18	13.99	1180	AT	X	0	X	X	Schultze (1975), Kral (1979)
Fehér-tó	46.47	21.16	86	HU	X	1	X	X	Magyari (2002), Sümegi *et al.* (2013)
Fiavè	46.01	10.84	654	IT	0	0	X	X	Grüger (1968)
Fornaci di Revine	46.05	12.26	224	IT	1	1	X	X	Casadoro *et al.* (1976); Kromer *et al.* (1998), Friedrich *et al.* (1999)
Gola di Lago	46.13	8.96	970	CH	0	Sc	X	Sc	Zoller & Kleiber (1971); Kral (1979)
Ioannina	39.75	20.85	470	GRE	X	0	X	X	Tzedakis (2000); Tzedakis et al. (2002)
La Grande Pile	47.73	6.50	330	FR	X	0	X	X	Woillard (1975, 1978); de Beaulieu and Reille (1992a)
Lac du Bouchet (D)	44.92	3.78	1200	FR	X	0	X	X	Reille & de Beaulieu (1990)
Lagaccione	42.57	42.57	355	IT	X	0	X	X	Follieri *et al.* (1998); Magri (1999)
Lago del Segrino	45.82	9.27	374	IT	X	0	X	0	Wick (1996)
Lago della costa	45.27	11.74	7	IT	X	1	X	X	Kaltenrieder *et al.* (2009), Kaltenrieder *et al.* (2010)
Lago di Annone	45.82	9.35	226	IT	X	1	X	0	Wick (1996); Vescovi (2007)
Lago di Biandronno	45.86	8.71	239	IT	X	1	X	0	Schneider (1978)
Lago di Gaiano	45.79	9.97	334	IT	X	1	X	0	Gehring (1997)
Lago di Ganna	45.98	8.82	452	IT	0	1	X	X	Schneider & Tobolski (1985)
Lago di Ledro	45.88	10.73	655	IT	X	1	X	X	Beug (1964); Kral (1979)
Lago di Lova	45.97	10.18	1299	IT	X	0	X	0	Gehring (1997)

Site	Latdd	Londd	Alt m a.s.l.	Country	M*	P*	Ch*	St*	Reference
Lago di Origlio	46.05	8.94	416	IT	X	1	X	0	Vescovi *et al.* (2007) ; Tinner *et al.* (1999)
Lago di Viverone	45.42	8.03	220	IT	X	1	X	1	Schneider (1978)
Lago Grande di Monticchio	40.93	15.58	656	IT	X	0	X	X	Allen *et al.* (1999)
Lago Lucone	45.55	10.48	249	IT	1	X	X	X	Valsecchi *et al.* (2006)
Lago Piccolo di Avigliana	45.06	7.38	353	IT	1	1	X	1	Finsinger *et al.* (2006); Finsinger & Tinner (2006); Vescovi *et al*. (2007)
Lake Banyoles	42.13	2.75	173	NA	X	0	X	X	Pérez-Obiol & Julia (1994)
Lake Brazi	45.40	22.90	1740	RO	0	1	X	1	Magyari *et al.* (2012)
Lake Galeş	45.39	22.91	1990	RO	X	1	X	X	Magyari *et al.* (2012)
Lake Xinias	39.05	22.27	500	NA	X	0	X	X	Bottema (1979)
Längsee	46.78	14.42	548	AT	X	1	X	X	Schmidt (1998)
Lanser See	47.24	11.42	840	AT	0	X	X	X	Oeggl (1992)
Leckermoor	47.81	14.93	877	AT	X	0	X	X	Bobek (1978); Kral (1979)
Linden	46.85	7.68	900	CH	X	0	X	X	Heeb & Welten (1972); Kral (1979)
MD95-2039	40.58	-10.35	-3381	NA	X	0	X	X	Roucoux *et al.* 2001
MD95-2043	36.14	-2.62	-1841	NA	X	0	X	X	Sánchez Goñi *et al.* (2002)
MD99-2331	41.15	-9.68	-2110	NA	X	0	X	X	Naughton *et al.* (2007)
Molkówka	49.28	19.82	956	PL	X	0	X	X	Koperowa (1962)
Nagymohos	48.41	20.41	394	HU	X	1	X	X	Magyari *et al.* (1999), Magyari (2002)
Navarrés	39.10	-0.68	225	NA	X	0	X	X	Carrión & Van Geel (1999)
Nowy Targ Basin	49.48	20.03	680	PL	X	0	X	X	Koperowa (1962)
ODP site 976	36.20	-4.30	-1108	NA	X	0	X	X	Combourieu-Nebout *et al.* (2002)
Padul	37.00	-3.07	795	ESP	X	0	X	X	Pons & Reille (1988)
Palù bei Edolo	46.18	10.33	660	IT	0	0	X	0	Gehring (1997)
Palughetto mire	46.10	12.40	1040	IT	0	1	X	0	Avigliano *et al.* (2000), Vescovi *et al.* (2007)
Pesteana	45.54	22.81	480	RO	X	1	X	X	Farcas *et al.* (2006)
Pian di Gembro	46.20	10.17	1350	IT	X	0	X	0	Pini (2002), Vescovi (2007)
Ribains	44.84	3.82	1192	FR	X	0	X	X	de Beaulieu and Reille (1992b)
Riparo Tagliente	45.52	11.02	250	IT	X	X	1	X	Maspero (1996)
Saltarino sotto	45.51	10.50	194	IT	1	1	X	X	Grüger (1968)
Simplon-Hobschensee	46.25	8.02	2017	CH	0	X	X	0	Lang & Tobolski (1985)
Simssee, Rosenheim	47.87	12.24	472	DE	X	0	X	X	Beug (1976), Kral (1979)
Sivárňa	49.32	20.58	610	SVK	X	1	X	X	Jankovská (1984, 1991, 2008)
Stracciacappa	42.13	12.32	220	NA	X	0	X	X	Giardini (2007)
Suossa	46.46	9.20	1700	CH	0	1	X	0	Zoller and Kleiber (1971)
Tenagi Philippon II	41.02	24.03	40	GRE	X	0	X	X	Wijmstra (1969)
Torbiera d'Alice Superiore	45.46	7.78	570	IT	X	1	X	0	Schneider (1978)
Torbiera di Castellaro	45.37	10.64	100	IT	X	0	X	X	Bertoldi (1968), Kral (1979)
Torbiera di Trana	45.06	7.42	360	IT	X	1	X	1	Schneider (1978)
Torfsee	45.49	7.89	270	IT	X	1	X	1	Schneider (1978)
Totenmoos	46.55	11.01	1718	IT	0	1	X	1	Heiss *et al.* (2005)
Trstenik-Gumance I	46.32	14.37	950	SLO	X	1	X	X	Sercelj (1971), Kral (1979)
Val Lastari	45.82	11.61	1060	IT	X	X	1	X	Maspero (1996)
Valle di Castiglione	41.88	12.76	44	IT	X	0	X	X	Follieri *et al.* (1988, 1998)
Wildmoos, Mondsee	47.78	13.39	800	AT	X	0	X	X	Bobek & Schmidt (1976), Kral (1979)

*1: presence, 0: absence, X: not investigated; M: Macrofossils, P: Pollen, Ch: Charcoals, St: Stomata

Table S 13 Fossil sites since 14.7 ka reported in the literature

Site	Latdd	Londd	Alt m a.s.l.	Reference
Lago di Ledro Garda	45.88	10.73	655	Beug (1964), Kral (1979)
Ballmoos	47.54	9.49	943	Kral (1979)
Buchensee	47.77	8.98	430	Bertsch (1961), Kral (1979)
Campra Lukmanier	46.53	8.87	1420	Müller (1972)
Etrach	47.18	13.99	1180	Schultze (1975), Kral (1979)
Gola di Lago	46.13	8.96	970	Zoller & Kleiber (1971), Kral (1979)
Leckermoor	47.81	14.93	877	Bobek (1978), Kral (1979)
Linden	46.85	7.68	900	Kral (1979)
Signater Kopf I	46.52	11.42	1260	Schmidt (1975), Kral (1979)
Simssee	47.87	12.24	472	Kral (1979)
Suossa	46.46	9.20	1700	Zoller and Kleiber (1971), Kral (1979)
Torbiera d'Alice Superiore	45.46	7.78	570	Schneider (1978)
Torbiera di Castellaro	45.37	10.64	100	Bertoldi (1968), Kral (1979)
Torbiera di Trana	45.06	7.42	360	Schneider (1978)
Trstenik-Gumance I	46.32	14.37	950	Sercelj (1971), Kral (1979)
Wildmoos	47.78	13.39	800	Bobek & Schmidt (1976), Kral (1979)
Bondone	46.03	11.04	1550	Grüger (1968)
Fiavè	46.01	10.84	654	Grüger (1968)
Saltarino sotto	45.51	10.50	194	Grüger (1968)
Gerzensee	46.83	7.55	603	Wick (2000)
Hérémence	46.15	7.37	2290	Wick (2000)
Regenmoos	46.62	7.39	1360	Wick (2000)
Maloja Riegel_4	46.41	9.69	1865	Ilyashuk *et al.* (2009)
Lago di Biandronno	45.86	8.71	239	Schneider (1978)
Lanser See	47.24	11.42	840	Oeggl (1992)
Torfsee	45.49	7.89	270	Schneider (1978)
Lago del Segrino	45.82	9.27	374	Wick (1996)
Palughetto mire	46.10	12.40	1040	Vescovi *et al.* (2007), Avigliano *et al.* (2000)
Zeneggen	46.28	7.84	1530	Tobolski & Amman (2000)
Leysin	46.20	7.01	1230	Wick (2000)
Lake Brazi	45.40	22.90	1740	Magyari *et al.* (2012)
Lake Galeş	45.39	22.91	1990	Magyari *et al.* (2012)
Lake Bled	46.37	14.27	475	Andrič *et al.* (2009)
Ley da Champfèr	46.47	9.82	1791	Gobet *et al*. (2005)
Jasło	49.75	21.47	250	Harmata (1995)
Lago Piccolo di Avigliana	45.06	7.38	353	Finsinger *et al*. (2006), Finsinger and Tinner (2006), Vescovi *et al.* (2007)
Brzeźnica C	50.10	21.48	130	Mamakowa & Starkel (1974)
Forcellona	46.01	11.76	1330	Kral (1980)
Podgrodzie	50.01	21.35	220	Mamakowa & Starkel (1977)
Lago di Lova	45.97	10.18	1299	Gehring (1997)
Preluca Tiganului	47.81	23.53	730	Feurdean & Bennike (2004), Wohlfahrt *et al.* (2001)
Lago di Annone	45.82	9.35	226	Wick (1996), Vescovi (2007)
Tarnowiec	49.70	21.61	220	Harmata (1987)
Lac de Fully	46.17	7.09	2135	Finsinger & Tinner (2007)
Tarnawa Wyzna	49.10	22.83	670	Ralska-Jasiewiczowa (1980)
Gouillé Rion	46.16	7.36	2343	Kaltenrieder *et al.* (2005)
Simplon_Alter Spitel	46.23	8.01	1885	Welten (1982a), van der Knaap & Ammann (1997)
Lago Basso	46.51	9.30	2250	Wick & Tinner (1997)
Grodzisko Nowe	50.50	22.45	200	Kolaczek (2010)
Dossaccio Bormio	46.28	10.20	1730	Welten (1982b), van der Knaap & Ammann (1997)
Capatana	46.51	23.15	1600	Farcas *et al.* (2005)
Längsee	46.78	14.42	548	Schmidt (1998)
Lago di Gaiano	45.79	9.97	334	Gehring (1997)
Lago di Ganna	45.98	8.82	452	Schneider & Tobolski (1985)
Lago di Origlio	46.05	8.94	416	Vescovi *et al.* (2007), Tinner *et al.* (1999)
Lago di Viverone	45.42	8.03	220	Schneider (1978)
Molkówka	49.28	19.82	956	Koperowa (1962)
Nowy Targ Basin	49.48	20.03	680	Koperowa (1962)
Sivárňa	49.32	20.58	610	Jankovská (1984, 1991, 2008), Kuneš *et al*. (2008)

Site	Latdd	Londd	Alt m a.s.l.	Reference
Totenmoos	46.55	11.01	1718	Heiss *et al.* (2005)
Pian di Gembro	46.20	10.17	1350	Pini (2002), Vescovi *et al.* (2007)
Simplon_Hobschensee	46.25	8.02	2017	Lang & Tobolski (1985)
Palù bei Edolo	46.18	10.33	660	Gehring (1997)
Luci	46.27	25.75	1079	Tantau *et al.* (2003), Feurdean *et al.* (2007)
Taul Zanoguti	45.33	22.80	1840	Farcas *et al.* (1999), Feurdean *et al.* (2007)
Col di Val Bighera	46.28	10.31	2087	Gehring (1997)
Passo del Tonale	46.31	10.58	1883	Gehring (1997)
Kis-Mohos Tó	48.41	20.41	310	Willis *et al.* (1997), Willis *et al.* (2000)
Jezor-Jaworzno	50.24	19.20	255	Szczepanek & Stachowicz-Rybka (2004)
Alpe de Venosc	44.99	6.12	1644	Coûteaux (1962), Kral (1979)
Autertal	46.86	13.93	1450	Fritz (1964), Kral (1979)
Boecklweiher	47.64	12.96	610	Kral (1979)
Brigels	46.77	9.06	1520	Müller (1972)
Edlbacher Moor	47.73	14.34	610	Kral (1979)
Feichtauer Moor	47.80	14.32	1340	Kral (1979)
Fusine	46.50	13.65	920	Kral (1982)
Kendlmühlfilz	47.80	12.44	525	Schmeidl (1977), Kral (1979)
Keutschachersee	46.59	14.16	508	Kral (1979)
Laghetto di Somdogna	46.47	13.37	1442	Kral (1982)
Malga di Lussari	46.50	13.55	1554	Kral (1982)
Moosham	47.66	13.79	1030	Kral (1979)
Poelland	46.63	13.53	1050	Fritz (1973), Kral (1979)
Pokljuka Sijec Julische Alpen	46.36	14.04	1170	Sercelj (1971), Kral (1979)
Rödschitzer Moos	47.56	13.92	775	Kral (1979)
Seemoos	47.08	13.78	1700	Bortenschlager (1964), Kral (1979)
Segner	46.89	9.19	1880	Müller (1972), Kral (1979)
Sur Oberhalbstein	46.52	9.63	1780	Heitz (1975), Kral (1979)
Tourbière d'Hinter-Hoehi	47.17	9.16	1420	Hoffmann & Grobety (1968), Kral (1979)
Wansenmoos beim Zellhof	48.01	13.10	500	Krisai (1975), Kral (1979)
Wiegenwald Stubachtal	47.22	12.61	1720	Kral (1979)
Baumgartl	47.50	12.97	1720	Mayer (1966), Kral (1979)
Cavazzo	46.37	13.04	270	Kral (1979)
Cavazzo-Vuarbes	46.38	13.02	270	Kral (1982)
Eben	47.42	13.39	850	Kral (1979)
Egelsee ob Diemtigen	46.63	7.55	1000	Welten (1952), Kral (1979)
Malga Varmost	46.51	12.59	1480	Kral (1982)
Puergschachener Moor	47.58	14.33	632	Kral (1979)
Schwimmend Moos	47.68	12.87	1370	Mayer (1966), Kral (1979)
Seekarmoor	47.25	13.39	1785	Kral (1979)
Weidfilz	47.78	11.43	595	Gross (1965), Kral (1979)
Lago Perso	44.91	6.80	2000	Blarquez et al. (2010)
Passo di Pramollo	46.56	13.28	1551	Kral (1982)
Pieve Tesino	46.04	11.81	1240	Kral (1980)
Hirschbichl	46.87	12.36	2140	Oeggl & Wahlmüller (1994)
Lac du Loup	45.19	6.54	2035	Blarquez *et al.* (2009)
Lej da San Murezzan	46.49	9.84	1768	Gobet et al. (2005)
Gouillé Loéré	46.15	7.36	2503	Tinner and Theurillat (2003)
Lengi Egga	46.36	7.96	2557	Tinner and Theurillat (2003)

List 1 *Larix* pollen sites from databases

Sources: ALPADABA, PALYCZ (http://botany.natur.cuni.cz/palycz/),
EPD (Giesecke, Davis, Brewer *et al.* submitted)

Sites (Latdd/Londd; Alt m a.s.l.)

Älbi Flue (46.6/7.98; 1850), Aletschwald (46.39/8.03; 2017), Aletschwald PJ (46.39/8.03; 2017), Alpi di Robièi, Val Bavona (46.44/8.52; 1892), Amburnex hummock (46.54/6.23; 1375), Atemlöchermoos (46.94/11.02; 1790), Bachalpsee (46.67/8.02; 2265), Baldeggersee (47.2/8.27; 463), Bedrina I (46.48/8.77; 1235), Bedrina II (46.48/8.77; 1235), Belalp Ba I (46.39/7.98; 2330), Belalp IIF (46.38/7.98; 2290), Bezdonnoe (62.03/32.77; 121), Bibersee (47.21/8.47; 429), Bitsch-Naters (46.34/7.99; 1030), Bláto (49.04/15.19; 658), Bledowo Lake (52.55/20.67; 78), Bobrov (49.45/19.66; 620), Böhnigsee 1 (46.26/7.85; 2095), Broumovské steny (50.56/16.28; 654), Buntes Moor (47/11.13; 2285), Cervená louka (50.13/13.72; 339), Cervené blato (48.86/14.81; 477), Chernikhovo (53.42/26.43; 168), Clapeyret 68 (44.13/7.23; 2260), Cristol Lake (45/6.63; 2248), Darzlubie Forest (54.7/18.17; 40), Dolgoe (55.23/28.18; 173), Dortmunder Hütte (47.02/11; 1880), Dossaccio (46.47/10.34; 1730), Dossaccio I (46.47/10.34; 1730), Dossaccio II (46.47/10.34; 1730), Dura-Moor (46.64/11.46; 2080), Dürrenecksee-Moor 12 (47.17/13.87; 1700), Dvur Anšov (48.78/16.42; 179), Egelsee (47.61/12.17; 549), Eggen ob Blatten (46.37/7.99; 1645), Eggen ob Blatten 56 (46.37/7.99; 1645), Etang de la Gruère EGr2A (47.24/7.05; 1005), Etang de la Gruère EGr2G (47.24/7.05; 1005), Etang de Luissel (46.24/7.02; 540), Etang d'y Cor (46.31/7.48; 1500), Ezerisch (55.85/30; 165), Feld D2 (46.66/8; 2130), Franz Senn-Hütte (47.08/11.02; 2115), Fuchsschwanzmoos 1 (47.12/13.9; 1680), Gänsemoos (46.83/7.36; 795), Gerlos (47.24/12.13; 1590), Gerzensee (46.83/7.55; 603), Giering (47.47/12.35; 820), Gondo-Alpjen 2 (46.21/8.11; 1635), Gorno (50.85/20.83; 240), Grächen-See (46.2/7.85; 1710), Grächen-See (2), (46.2/7.85; 1710), Greicheralp (46.38/8.03; 1910), Grindjisee (46.01/7.79; 2334), Grosses Überling Schattseit M. (47.17/13.9; 1750), Grünsee (46.86/10.48; 1836), Hagelseeli-1 (46.67/8.04; 2339), Hasenmoos (47.47/12.38; 770), Hinterburgseeli (46.72/8.07; 1510), Höhenbiel (46.57/8.5; 1970), Hopschensee (46.25/8.02; 2017), Hopschensee top (46.25/8.02; 2017), Horní Lomná (49.52/18.63; 608), Hozelec (49.05/20.33; 685), Hroznotín (49.76/15.36; 520), Hyncice (50.62/16.29; 419), Il Fuorn (46.66/10.21; 1805), Jablunka (49.38/17.95; 322), Jammertal (48.1/9.73; 578), Jasiel (49.37/21.89; 680), Jaslo (49.78/21.47; 250), Jedlová (49.4/19.66; 660), Juf Plan (46.63/10.26; 2225), Kamenicky (49.74/15.96; 632), Katzenloch (47.33/11.12; 1220), Kepskoe (65.08/32.17; 124), Kirchbichl (47.5/12.09; 512), Klícava (50.15/13.83; 440), Královec (49.13/18.03; 599), Krinice (50.57/16.31; 390), Krotenweiher I (47.08/11.4; 1310), Kružlová (49.36/21.58; 0), Kubriková (49.48/18.67; 790), Kunštátská kaple (50.25/16.45; 1005), Labský dul (50.77/15.55; 1199), Lac d'Aï (46.36/7.01; 1891), Lac de Bretaye (46.33/7.07; 1780), Lac de Villa (45.68/7.76; 820), Lac du Mont d'Orge (46.23/7.34; 640), Lac Long Inférieur 1 (44.06/7.46; 2093), Lac Mouton (44.06/7.44; 2175), Lac Saint Léger (44.42/6.34; 1308), Laghi dell'Orgials (44.23/7.13; 2240), Lago di Bévera (45.85/8.89; 325), Lago di Ganna (45.9/8.83; 452), Lago Grande di Monticchio (40.94/15.6; 1326), Laguna de la Roya (42.22/-6.77; 1608), Lai Nair (46.78/10.31; 1546), Lake Gosciaz (52.58/19.35; 64), Lake Mikolajki (53.77/21.42; 116), Lake Racze (53.92/14.67; -9), Lanser Moor I (47.24/11.42; 840), Lanser Moor III (47.24/11.42; 840), Leysin, Les Léchières (46.21/7.01; 1255), Lindenmoos (47.51/12.04; 640), Liptovský Jan (49.04/19.68; 660), Loucky (49.32/15.53; 595), Machová (48.83/17.54; 468), Malschötscher Hotter (46.67/11.45; 2050), Martínkovice (50.55/16.34; 390), Mieminger See (47.28/10.98; 800),

Mittlere Hellelen (46.17/7.5; 1520), Mladotice (49.22/13.8; 633), Mont Carré (46.15/7.37; 2290), Moor Alpenrose (47.05/11.75; 1880), Moor am Rofenberg (46.87/10.82; 2760), Motta-Naluns (46.81/10.26; 2170), Mrtvý luh (48.87/13.88; 737), Nagymohos (48.41/20.41; 394), Nahorany (49.14/13.83; 659), Naroch (54.82/26.75; 165), Niechorze (54/15.05; 5), Oberaar G I 112 (46.55/8.26; 2315), Palašiny (49.68/15.48; 506), Pancavská louka (50.77/15.54; 1336), Pillermoos Untergurgl (46.9/11.03; 1780), Pillon (46.36/7.2; 1670), Plan du Laus (44.24/6.7; 2121), Plešné jezero (48.78/13.87; 1105), Plidutscha (46.64/8.68; 2128), Popradské pleso (49.16/20.14; 1494), Praha-Podbaba (50.11/14.39; 189), Pré Rond (44.92/6.59; 1800), Ptichje (66.35/30.57; 120), Puscizna Rekowianska (49.48/19.82; 656), Rásná (49.23/15.37; 696), Regetovka (49.43/21.28; 515), Režabinec (49.25/14.09; 368), Riffelsee (45.98/7.76; 2757), Rinderplatz (46.64/11.48; 1780), Robiei II (46.45/8.52; 1892), Rotmoos Obergurgl (46.83/11.02; 2260), Roztoki (49.72/21.58; 230), Sägistalsee (46.68/7.98; 1935), Schönwies (46.85/11.03; 2260), Schönwies I (46.85/11.03; 2260), Schöpfenwaldmoor (46.74/7.85; 1450), Schwarzsee (46.67/11.43; 2033), Schwarzsee FR (46.67/7.27; 1046), Schwarzsee ST (46.87/10.48; 1721), Schwarzsee VS (45.15/7.12; 2552), Schwemm A3 (47.65/12.3; 664), Schwemm B1 (47.65/12.3; 664), Schwemm B5 (47.65/12.3; 664), Schwemm D3 (47.65/12.3; 664), Schwemm F4 (47.65/12.3; 664), Seebergsee (46.58/7.44; 1831), Seefelder See (47.32/11.18; 1200), Selle di Carnino I (44.15/7.68; 1905), Simplon, Alte Spittel/Gampisch (46.23/8.01; 1885), Simplon-Alter Spittel (46.23/8.01; 1885), Slopiec (50.78/20.78; 248), Sommersüss (46.76/11.68; 870), Spišská Belá (49.18/20.45; 625), Steklin (52.93/18.98; 73), Štrbské pleso (49.14/20.04; 1346), Tarnawa Wyzna (49.1/22.83; 670), Tarnowiec (49.7/21.62; 220), Tauernmoos (47.17/12.64; 2100), Teplice nad Metují (50.59/16.17; 466), Tlstá hora (48.89/17.89; 460), Torbiera del Biecai (44.2/7.7; 1920), Tourbière de Champlong (45.82/7.81; 2320), Tourbière de Pilaz (45.82/7.83; 1460), Tourbière de Santa Anna (45.86/7.65; 2304), Trogenmoos (46.76/7.86; 1470), Umbrail (46.54/10.43; 2490), Úpské rašelinište (50.74/15.71; 1425), Ust'Mashevskoe (56.32/57.88; 220), Vacovice (49.14/13.79; 718), Vallon de Provence (44.39/6.4; 2075), Velký Ded (50.08/17.22; 1383), Vernérovice (50.62/16.2; 492), Vlcí rokle (50.6/16.13; 583), Voros-mocsar (46.48/19.19; 91), Wallbach I (46.43/7.4; 1885), Waxeckalm (47.03/11.08; 1875), Wildmoos (46.95/11.02; 1435), Wolbrom (50.38/19.77; 375), Woryty (53.75/20.2; 105), Xirès, Montana (46.31/7.47; 1445), Zarnowiec Peat Bog (54.72/18.12; 5), Zaruckoe (63.9/36.25; 20), Zbudovská blata (49.07/14.35; 396), Zeneggen-Hellelen A (46.28/7.84; 1520), Zeneggen-Hellelen B (46.28/7.84; 1520), Zirbenwaldmoor (46.86/11.03; 2150), Zlatnická Dolina (49.52/19.28; 850), Zotensenk I (47.42/11.87; 560), Zsombo Swamp (46.36/19.99; 92), Zuratkul' (54.9/59.27; 720).

List 2 *Larix* stomata sites from the EPD

Sites (Latdd/Londd; Alt m a.s.l.)

Aletschwald PJ (46.39/8.03; 2017), Bachalpsee (46.67/8.02; 2265), Böhnigsee 1 (46.26/7.85; 2095), Dossaccio (46.47/10.34; 1730), Eggen ob Blatten (46.37/7.99; 1645), Gondo-Alpjen 2 (46.21/8.11; 1635), Grächen-See (46.2/7.85; 1710), Greicheralp (46.38/8.03; 1910), Grünsee (46.86/10.48; 1836), Hopschensee (46.25/8.02; 2017), Lac Superieur de Fully (46.17/7.09; 2135), Lac du Mont d'Orge, Sion (46.14/7.2; 640), Lai Nair (46.78/10.31; 1546), Mont Carré (46.15/7.37; 2290), Robiei II (46.45/8.52; 1892), Simplon-Alter Spittel (46.23/8.01; 1885), Zeneggen-Hellelen (46.28/7.84; 1520)

CHAPTER 6: Synthesis and perspectives

In my PhD I precisely documented the consequences of past climate and anthropogenic changes in European larch populations working at multiple temporal and spatial scales and from different perspectives. Before this project information on the history of *Larix decidua* was very limited due to a lack of high resolution genetic data and of a detailed fossil data compilation. A thorough literature survey on existing genetic markers in European larch and considerations of future needs in larch conservation monitoring and traceability motivated the design of new nuclear microsatellites (Chapter 2 and 3). The highly informative markers I obtained could be applied successfully on a range-wide sample of 45 modern *Larix* populations. In the genetic analysis I first concentrated on recent plantation history. I could establish a baseline for future studies dealing with translocations (Chapter 4) and reconstruct the ancient genetic structure to be compared with paleontological evidence. In the paleontological part of my work I focused on a detailed range-wide and chronologically precise compilation that could be correlated to past climate and anthropogenic events, albeit I could only briefly address the latter point (Chapter 5). The wealth of precise genetic and paleobotanical data provides a sound basis to discuss the origin of ancient genetic structure of modern *Larix decidua* populations and to evaluate past climate and anthropogenic impact.

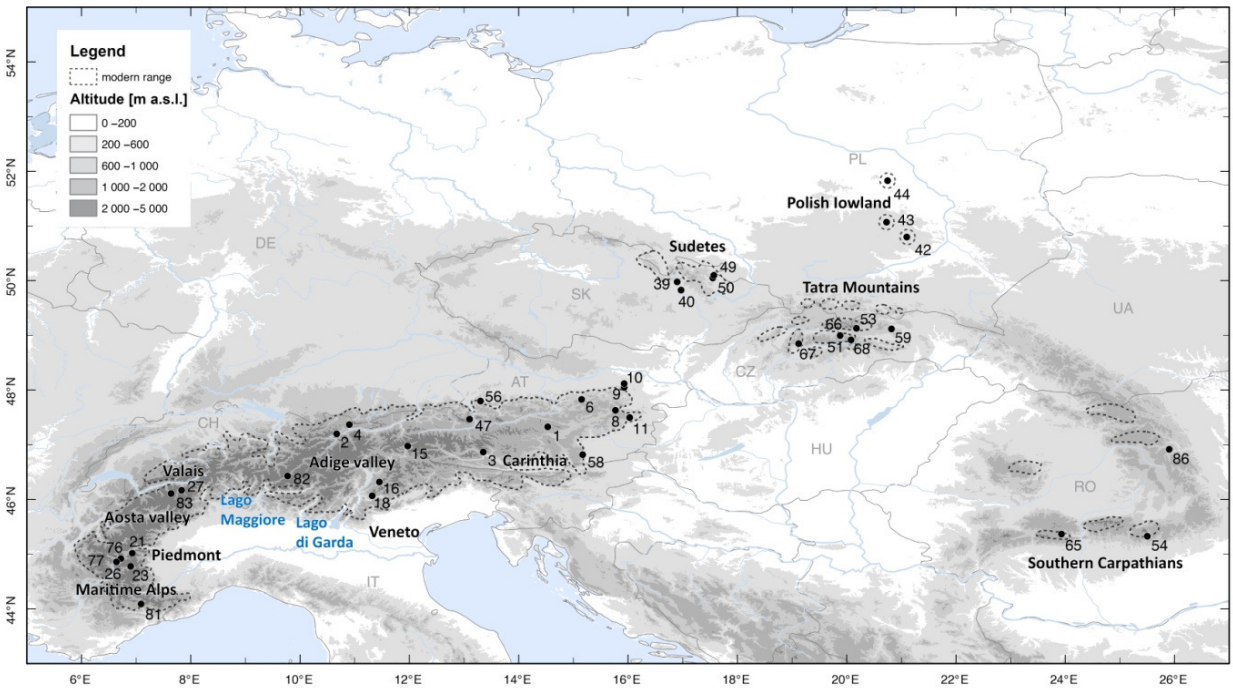

Figure 20 Studied modern larch populations and names of geographic locations cited in the text

Ancient genetic structure

The study uncovered an exceptionally strong genetic structure using both nuclear and mitochondrial DNA markers in the European larch (see CHAPTER 4). Seven nuclear genetic clusters have been identified (Fig. 21): four in the Alpine region and three in the Central European region. The genetic distance between nuclear clusters from these two regions is particularly large. The analysis of the mitochondrial genome, which evolves very slowly in plants including Conifers (Hipkins & Krutovskii 1994), also indicates a clear separation between Alpine and Central European lineages. Altogether, this suggests that the split between Alpine and Central European populations is very ancient. In the Alpine region, the four nuclear clusters as well as the two most common mitochondrial haplotypes (haplotypes 16 and 18) are distributed longitudinally. Moreover, the mitochondrial haplotypes alternate along the same direction (with the following sequence of haplotypes from west to east: 16/18/16/18). In most cases, nuclear clusters are characterized by one major mitochondrial haplotype (nuclear cluster 1 with haplotype 16, cluster 2 with haplotype 18, cluster 3 with haplotype 16, cluster 4 with haplotype 18), indicating that there has been no important gene flow, whether by seed or by pollen, between the corresponding groups of populations. However, we found two populations in the Swiss central Alps in the region of Valais (populations 27 and 83) that depart from this pattern. These populations were assigned to nuclear cluster 1 and carry haplotype 18. This suggests that the bulk of the ancestors of these populations originate from the southwestern Alps but have become introgressed by a mitochondrial haplotype originating from a refuge located further east, in the Venetian region. Another important result was the identification of a rare haplotype (haplotype 22) only found in the Maritime Alps and slightly further to the north (fixed in population 81 and mixed with haplotype 16 in population 77). This pattern could suggest a separate glacial refugia in the Maritime Alps. Yet, at nuclear markers, these two populations were both assigned to cluster 1 and did not differ from neighbouring populations fixed for mitochondrial haplotype 16, suggesting that either divergence within the two nearby refugia was restricted to mitochondrial DNA or that there has been subsequent genetic homogenisation through gene flow in the region.

In Central Europe we found one nuclear cluster in the Sudetes Mountains (cluster 5), one in the Tatra Mountains (6) and one in the eastern and southern Carpathians (7). In the Polish lowland nuclear assignment was less clear as clusters from the Sudetes and the Tatra Mountains co-occurred. In addition there were genotypes with intermediate assignments in

this part of the range. One mitochondrial DNA haplotype (number 8) was endemic to this area, though found in only one of the three sampled populations (population 44). This may indicate that Polish lowland populations originate from a different source than the Tatra and Sudetes populations. Alternatively, these populations could result from admixture between Tatra and Sudetes populations. To evaluate these hypotheses, new investigations are needed.

Refuges

To reconstruct refuges and migrational pathways, modern nuclear and mitochondrial DNA genetic structure can be used together with the fossil data. Early fossil evidence from the LGM and the subsequent Heinrich event 1 suggests that at least six of the seven nuclear clusters originate from distinct refuges: cluster 1 from the Turin refuge, cluster 2 from the Venetian refuge, cluster 3 from a southern Carinthian refuge, cluster 5 from the Sudetes refuge, cluster 6 from the western Carpathians refuge and cluster 7 from the southern Carpathians refuge. No fossil data is available that points to a refuge corresponding to nuclear cluster 4. It seems likely that this cluster also originates from a separate refuge as the eastern Alpine margin provided favourable environments during the LGM where endemic herbaceous taxa associated to upper montane coniferous forests persisted (Tribsch & Schönswetter 2003). The rare mitochondrial DNA haplotype found in the Maritime Alps seems to indicate the existence of another refuge that is also reflected in the early but limited fossil evidence found there before the Bølling/Allerød period at ~16.2 ka. Finally, both nuclear and mitochondrial data point to a distinct origin of populations from the Sudetes and from the Carpathians and hence corroborate evidence given by the fossils.

Colonization pathways

Fossils, nuclear and mitochondrial data indicate that recolonization of the southwestern Alps mainly involved source populations located in the Turin region. In addition there may have been some colonization from the presumed Maritime Alps refuge. For the central Swiss Alps data suggest colonization from the south. It is possible that the presumed introgression took place during the time when populations were abundant in the lowlands (LGM – Bølling/Allerød). Indeed, the strong biogeographical barrier in the Aosta zone (Thiel-Egenter *et al.* 2011) makes it unlikely that introgression occurred after the establishment of subalpine

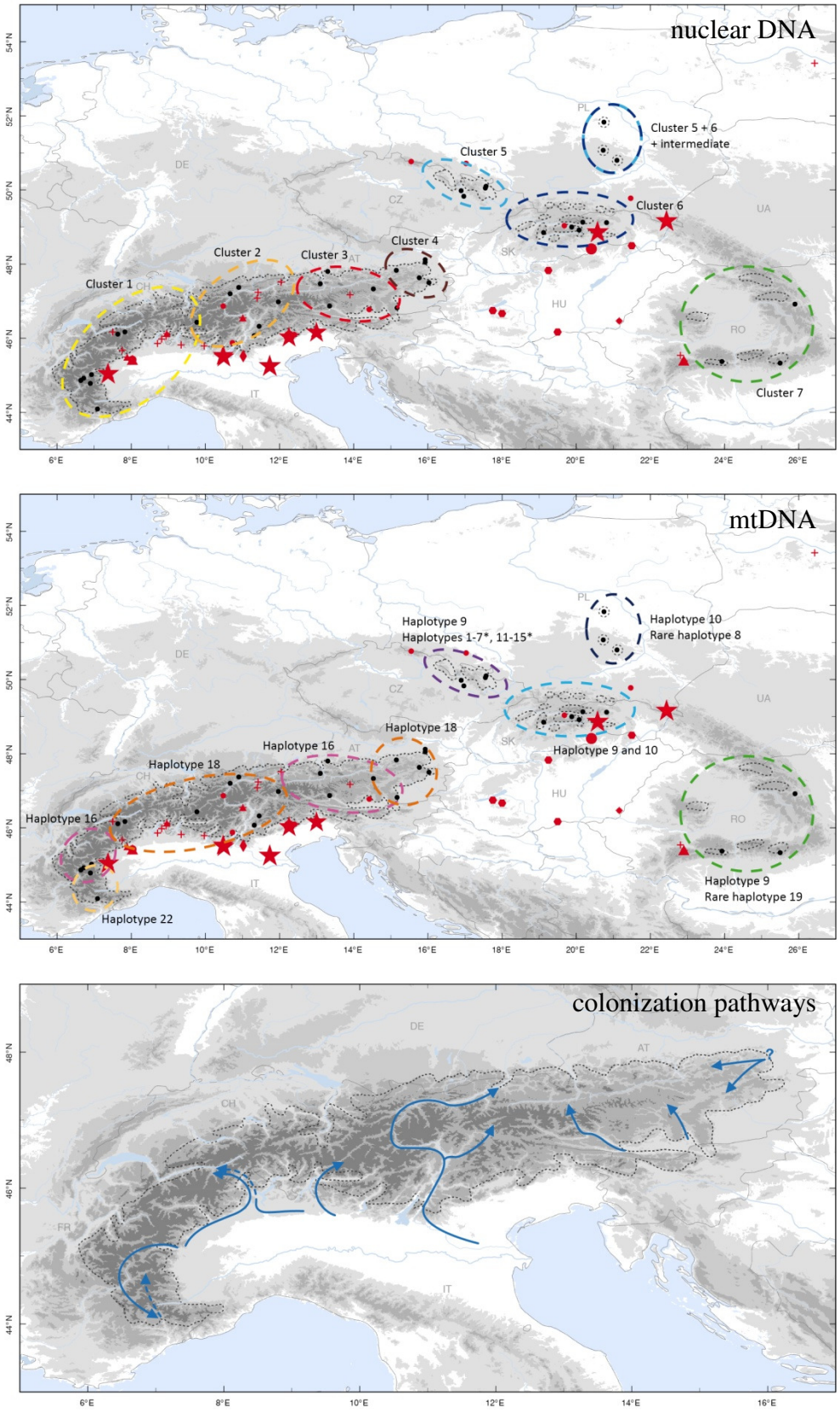

Figure 21 Ancient genetic structure at nuclear microsatellites and mtDNA, and colonization pathways. Red symbols represent early fossil evidence (23.5 – 14.7 ka). Black dots correspond to studied modern populations. Haplotypes with an asterisk are minisatellite variants. Dashed line symbolizes introgression of mtDNA.

forests. In the region located between the Lago Maggiore and Lake Garda, fossil and genetic data suggest a re-colonization from the southern Alpine foreland with the Venetian and Trentino-Alto-Adige region as the most important source area. Migration across the Alps might have followed the Adige-Inn valley. In fact, the Adige zone represents a second important contact zone (Thiel-Egenter *et al.* 2011). This is testified by a genetic split of larch populations. Westwards to the Adige Valley, migrational pathways cannot be inferred in such a detail due to scarcity of the fossil data. However, migration in different directions from the refuge in southern Carinthia seems likely.

In Central Europe modern larch populations should originate mainly from upward shifts from refuges in the Sudetes, the northwestern Carpathians and the southern Carpathians. The lowland populations from central Poland are (slightly) genetically distinct from the Tatra and Sudetes populations and may originate from populations persisting north to the western Carpathians. However, more investigations will be necessary to conclude about this issue.

Climatic and anthropogenic impacts

The paleontological and genetic methodologies combined in this study allow evaluating climate and anthropogenic effects on history of European larch at multiple temporal and spatial scales and from different perspectives. The genetic approach allows historical insights but only from the perspective of persisting modern populations. It indicates that modern distribution of genetic lineages is explained on the one hand by climate (ancient genetic structure) and on the other hand by recent translocations (in superposition to the ancient genetic structure). To evaluate when anthropogenic changes became superimposed to climate changes, fossil data are necessary. For larch, fossils clearly show that populations were impacted by anthropogenic changes (e.g. grazing, burning, cutting) since the Bronze Age, as judged from shifts in pollen and macrofossil abundance in populations at different altitudes from different parts of the Alpine and Carpathian range (e.g. Feurdean & Willis 2008; Kaltenrieder *et al.* 2005; Schneider & Tobolski 1985). In the Swiss Alps for instance stable subalpine forests with larch became replaced by meadows and shrublands due to contemporaneous climatic and anthropogenic changes whereas in a low altitudinal Italian site larch became favoured when cultivation of *Juglans* and *Castanea* started, confirming the predominant anthropogenic influence. In some cases fossil data can even confirm and complement genetic evidence of recent translocations by providing a time scale. In Slovakia

and the Czech Republic for instance, larch fossils become abundant 500 years ago in several low elevation sites clearly located beyond its natural range (Fig. 19). To date, inferring such time frame using genetic data alone is not possible.

Important processes that can only be investigated using a genetic perspective are introgression and admixture, which are increasingly endangering native plant and animal populations worldwide (Allendorf *et al.* 2001). In European larch human-induced admixture has been shown to be a frequent phenomenon, in particular in Slovakia, the Czech Republic and Poland. In some populations, including populations of the red list variant "*polonica*", admixture rates are alarming. Furthermore, as larch provenances differ in sensitivity towards larch canker, translocation of maladapted provenances can lead to population decline (Münch 1936). For instance, in France, larch canker has become a serious problem in larch plantations, both within and beyond the natural distribution range. In a related study, I applied the markers developed in chapter 3 to trace the origin of planted larch populations. I could show that the provenances that were most sensitive originate from the western Alps, which is in agreement with indications given by larch provenance tests (see Appendix n°1). This demonstrates that the markers developed in this study, along with the range-wide reference sample, can be used to answer practical questions in forestry.

These results show that to understand anthropogenic impact on tree populations, both fossil and genetic data are necessary. Fossil data allow to go further back in time and to understand the timing of human influences whereas genetic data allow studying translocation events in more detail (source, relative abundance and degree of admixture). Inference on the individual impact of specific long-term and short-term climate oscillations cannot be made based on modern genetics as it is not possible to correlate genetic changes to climate changes on the timescale of millennia. In fact, a lot of information on climate impact potentially registered in DNA has become lost due to subsequent population extinctions. Alongside with these intrinsic constraints, there are technical and methodological gaps in terms of genetic data acquisition and genetic data analysis. Fossils allowed us to precisely trace *Larix* responses to long-term trends and short-term climate events and revealed multiple range changes as well as shifts in species local abundance. The fact that six out of seven refuges could be identified with fossils confirms the high quality of the fossil compilation. The detailed historical inferences on recent and ancient history of European larch based on combined and complementary genetic and paleontological results can be seen as a new step in reconstructing tree history.

Perspectives

Using modern population samples and fossil data on finer spatial and temporal scales will advance our knowledge on processes related to translocations, an important task that has been neglected so far. The improved understanding of European larch history and the accurate description of its genetic structure at presumably neutral molecular markers provide a baseline for investigations on adaptation and selection in the context of future climate change. It can also represent a starting point for forthcoming palaeogenetic studies that could focus on the genetic impact of rapid past climate events.

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APPENDIX 1

Le chancre du mélèze dans le Massif Central en fonction de l'origine génétique des peuplements

Dominique PIOU, Stefanie WAGNER, Olivier FABREGUETTES, Cécile ROBIN

L'enquête lancée par le DSF en 2012 pour caractériser la fréquence et la sévérité des attaques de chancre dans 55 peuplements de mélèze du Massif Central n'a pas permis de mettre en évidence une différence de sensibilité entre les reboisements réalisés avec des provenances Sudètes ou d'Europe de l'Est et ceux réalisés avec des provenances ouest-européennes. Cette absence d'effet est en contradiction avec les études conduites en Europe depuis 1944 sur la variabilité naturelle du mélèze d'Europe (1ère et 2ème expériences internationales IUFRO) qui ont confirmé que les populations d'Europe de l'ouest sont plus sensibles à la maladie que celles d'Europe centrale (Schober, 1985). Sylvestre-Guinot et Delatour (1983) ont par ailleurs démontré expérimentalement que les provenances alpines ont un moins bon comportement à l'inoculation artificielle que celles d'Europe de l'Est.

Pour expliquer cette contradiction, nous avons fait l'hypothèse que les certificats de provenance recueillis par les gestionnaires avant chaque reboisement et conservés dans les archives pouvaient comporter des erreurs et qu'ils ne correspondaient pas forcément à l'origine exacte du matériel génétique utilisé. Pour tester cette hypothèse, nous avons utilisé les marqueurs microsatellites spécifiques du Mélèze d'Europe mis au point par Wagner et al (2012), qui permettent de différencier 7 grandes régions de provenance à l'intérieur de l'aire naturelle de *Larix decidua*. (Annexe 1). Les premiers résultats encore provisoires sont présentés ci après.

Matériel et méthodes

Choix des peuplements :

En 2012, les recherches de provenances à l'aide de marqueurs microsatellites ont été réalisées sur 192 arbres provenant de 39 peuplements, choisis essentiellement parmi ceux pour lesquels nous possédions des mentions "administratives" de provenance. L'annexe 3 fournit le détail du nombre d'arbres échantillonnés par peuplement et la figure 1 la répartition des peuplements échantillonnés. Les peuplements ont été répartis en trois catégories en fonction de leur origine supposée d'après les documents administratifs : ALP adm (peuplements issus de provenance alpine ou de peuplements français classés), CE adm (peuplements issus de provenance d'Europe centrale), et NA adm (non affectés). Les autres peuplements de l'enquête DSF seront analysés en 2013.

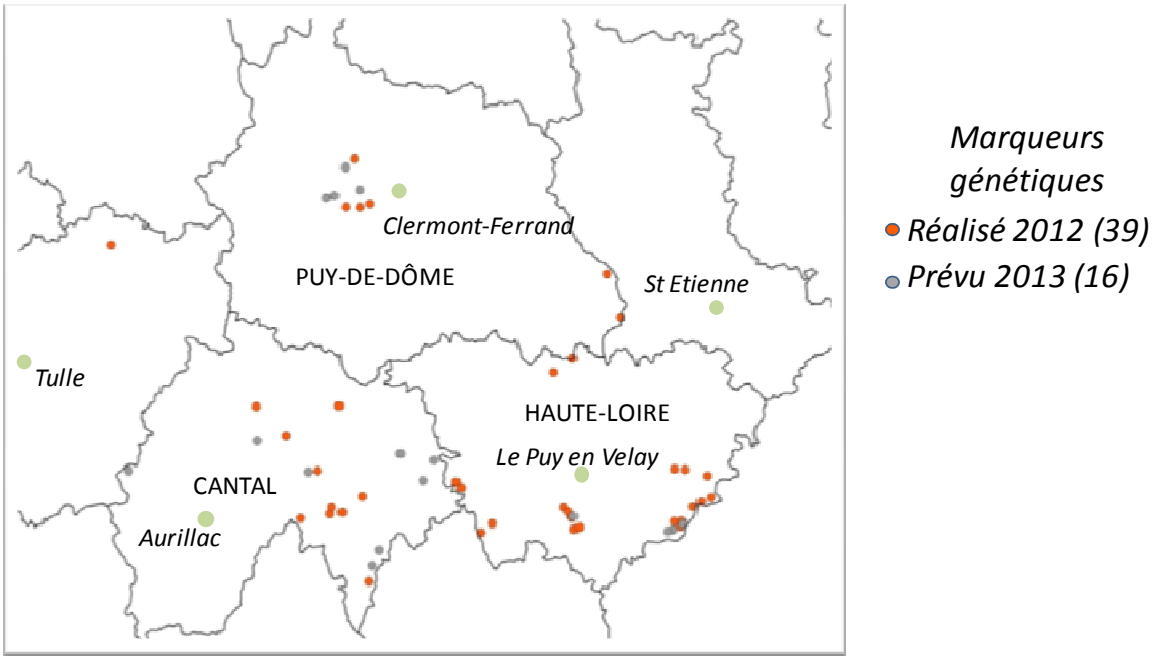

Figure 1 – Répartition géographique des peuplements de Mélèze dont l'origine génétique a été recherchée par marqueurs**.**

Echantillonnage :

Des aiguilles de mélèze ont été récoltées entre le 14 juin et le 23 juillet 2012, sur cinq arbres par peuplement (4 arbres pour le peuplement 11987, et 3 pour 12647) et déshydratées immédiatement dans du silica gel. L'effectif de cinq arbres a été choisi du fait des contraintes techniques et aussi au vu du fort apparentement génétique intra-peuplements observés sur l'ensemble de l'aire naturelle du mélèze *L . decidua* (S. Wagner, unpublished).

Extraction d'ADN

L'ADN de mélèze a été extrait avec le kit *DNeasy Plant Mini Kit* de la compagnie Qiagen. Deux à trois aiguilles découpées en morceaux de 2mm ont été utilisées pour chaque échantillon et broyées (billes de 2mm de diamètre) à pleine puissance dans un Mixer Mill. Les concentrations des solutions d'ADN obtenues ont été ramenées à 10 ng/µl avant amplification par PCR.

Génotypage et assignation des individus

Les microsatellites et multiplex mis au point par Wagner *et al.* (2012) ont été utilisés dans les conditions décrites par ces auteurs (95°C 15mn, 35 ou 30 cycles à 94°C, 56°C, ou 72°C 1mn, et incubation finale à 60°C 30mn). Les multiplex 1 et 2 sont composés respectivement de 7 et 6 couples d'amorces. Le génotypage a été réalisé sur la Plateforme Génome-Transcriptome de Bordeaux, avec un séquenceur ABI 370 (Applied Systems, USA). Des génotypes de *L. decidua* de référence utilisés pour la mise au point des amorces et multiplex ont été génotypés en même temps que les individus étudiés et ont été utilisés comme témoin pour la lecture des chromatogrammes et la notation des allèles. Celles-ci ont été faites à l'aide du logiciel Strand (Wagner *et al.* 2012).

En étudiant 45 provenances réparties sur toute l'aire de distribution du mélèze et en utilisant les programmes STRUCTURE (Pritchard *et al*. 2000) qui met en œuvre une méthode bayésienne de clustérisation, S. Wagner a mis en évidence au cours de sa thèse l'existence de sept pools génétiques chez le mélèze (W Alps, C alps, CE Alps, E Alps, Sudètes, Tatra, S Carpathes, cf annexe 1). En fait ces sept clusters peuvent être regroupés en deux pools génétiques, fortement différenciés : Alpes et Europe de l'ouest (ALP) et Europe centrale (CE). Les individus du Massif Central représentant les 39 peuplements étudiés ont été rajoutés à ce jeu de données afin de déterminer leur origine génétique. Chaque individu a pu être ainsi caractérisé par une probabilité d'affectation à l'origine Alpine (p_A, de 1 à 0) et à l'origine Europe centrale (p_{CE}=1-p_A). Si une de ces probabilités est supérieure à 0.875, l'individu est affecté à la zone de provenance correspondante. Pour les valeurs inférieures à ce seuil de p_A et p_{CE}, l'individu est déclaré « intermédiaire » (I). Si 75% au moins des individus testés d'un peuplement sont assignés à une même zone de provenance (ALP ou CE) ou à une même catégorie (I), on considère qu'ils représentent l'origine la plus probable de tous les arbres du peuplement. Dans le cas contraire le peuplement est qualifié de peuplement mélangé (M).

Résultats

Diversité et origine génétique des peuplements étudiés :

Le nombre d'allèles observés pour chaque locus varie entre 8 et 26, en accord avec la diversité allélique déjà décrite chez *L. decidua* (cf Table 1). Pour deux loci, le nombre d'allèles observés est même supérieur à celui décrit. Il y a très peu de données manquantes (0.84%).

Tableau 1 : Richesse allélique observée à l'intérieur des populations du Massif Central

multiplex	Couple d'amorces	nb allèles observés	nb allèles décrits*
1	bcLK253	14	17
1	bcLK211	20	28
1	bcLK228	20	15
1	Ld30	15	17
1	Ld31	16	23
1	Ld50	15	19
1	bcLK189	14	15
2	Ld101	12	15
2	Ld42	8	9
2	Ld56	13	14
2	Ld58	20	25
2	bcLK263	26	36
2	Ld45	13	12

* : in Wagner *et al.;* 2012

Soixante-trois et 54 arbres ont pu être assignés respectivement aux origines ALP et CE (cf annexe 2). Par contre 75 individus (plus du tiers) ont des probabilités intermédiaires (Fig. 2B). En comparaison, en se basant sur les certificats de provenance et en considérant que tous les arbres d'un même peuplement proviennent d'une seule et même région, 25 et 152 arbres censés correspondre à des provenances ALP et CE et 25 sont de provenance inconnue (Fig 2A). On constate donc une forte incohérence entre ces deux résultats.

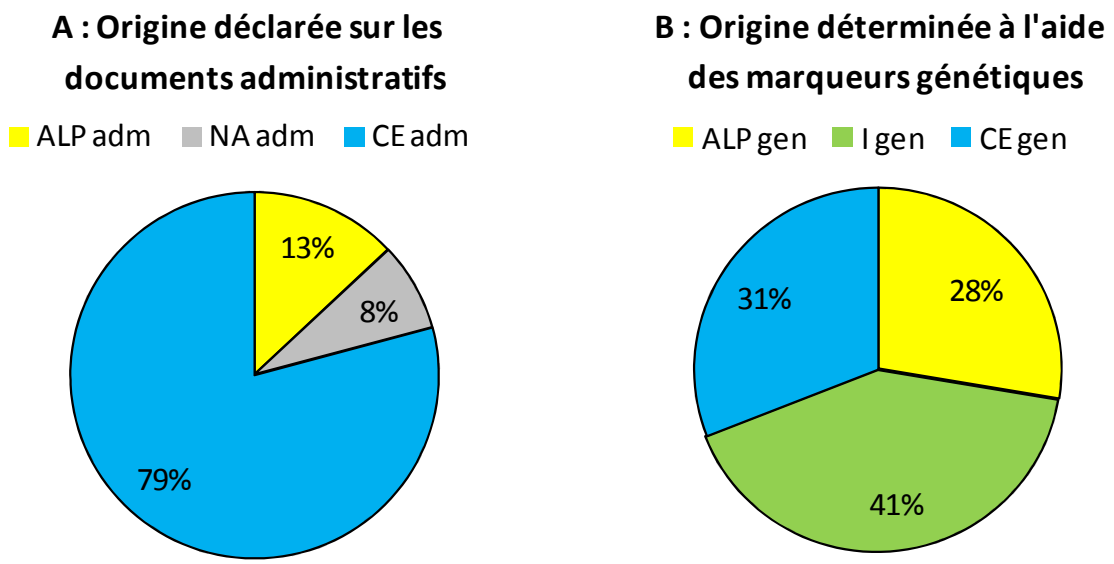

Figure 2. Distribution comparée des 192 mélèzes en fonction de leur origine (tous peuplements confondus). ALP : origine Alpes, CE : origine Europe centrale, NA : non documenté ; I : individus intermédiaires.

La figure 3 propose la comparaison croisée pour tous les arbres, entre les informations issues des documents administratifs et celles provenant des marqueurs génétiques. Il apparaît que chaque groupe "administratif" de provenance comprend des arbres appartenant aux trois classes de marqueurs génétiques. Le groupe des mélèzes rattachés administrativement à l'Europe centrale, le plus nombreux, comptent en réalité 30,9% d'arbres effectivement d'Europe centrale sur la base des marqueurs, 27,6% d'arbres d'origine alpine et 41,4% d'arbres classés parmi les intermédiaires. Le groupe rattaché aux Alpes comprend une majorité (64%) de mélèzes effectivement d'origine alpine d'après les microsatellites.

La proportion de mélèzes originaire d'Europe centrale est plus importante (33%) dans les peuplements âgés de 6-10 ans que dans ceux âgés de 15-25 ans (20%). Cette différence est à la limite de la significativité (Mann-Whitney ; z=-1,94 ; p = 0,053).

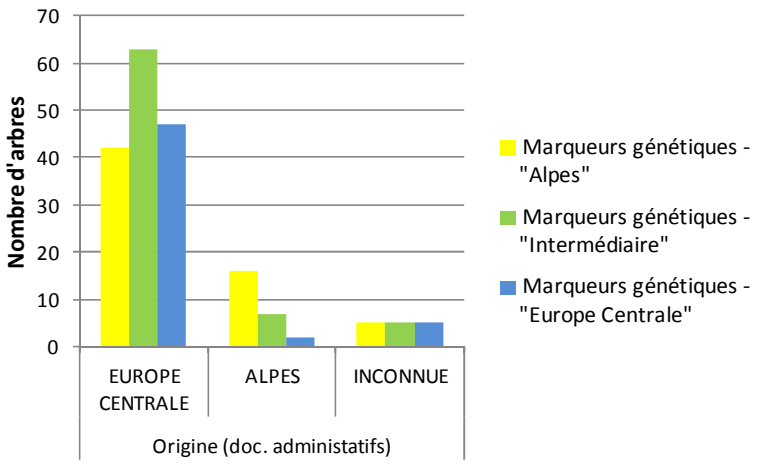

Figure 3 : Distribution croisée de l'origine des 192 mélèzes (tous peuplements confondus) en fonction des informations déclarées sur les documents administratifs et des marqueurs génétiques

En fonction des assignations individuelles, l'origine la plus probable de chacun des peuplements étudiés a été identifiée (Fig. 4). Dans sept peuplements, quatre ou cinq arbres ont une origine alpine et sont donc considérés comme provenant des Alpes. Suivant cette règle, cinq peuplements ont une origine Europe centrale et sept sont composés d'individus intermédiaires. Dans les autres peuplements (19 parmi 39, peuplements M), aucune origine ou catégorie n'est dominante.

Il apparaît clairement (Fig.5) que parmi les peuplements déclarés d'Europe centrale sur la base des certificats de provenance, une majorité correspond en réalité à un mélange d'arbres assignés aux populations alpines et d'Europe centrale. Les peuplements "purs" en mélèzes d'Europe centrale sont beaucoup moins nombreux qu'attendus d'après les documents administratifs.

De même sur les 5 peuplements "alpins" ou d'Europe de l'ouest d'après les documents administratifs, 2 seulement apparaissent purs pour cette provenance.

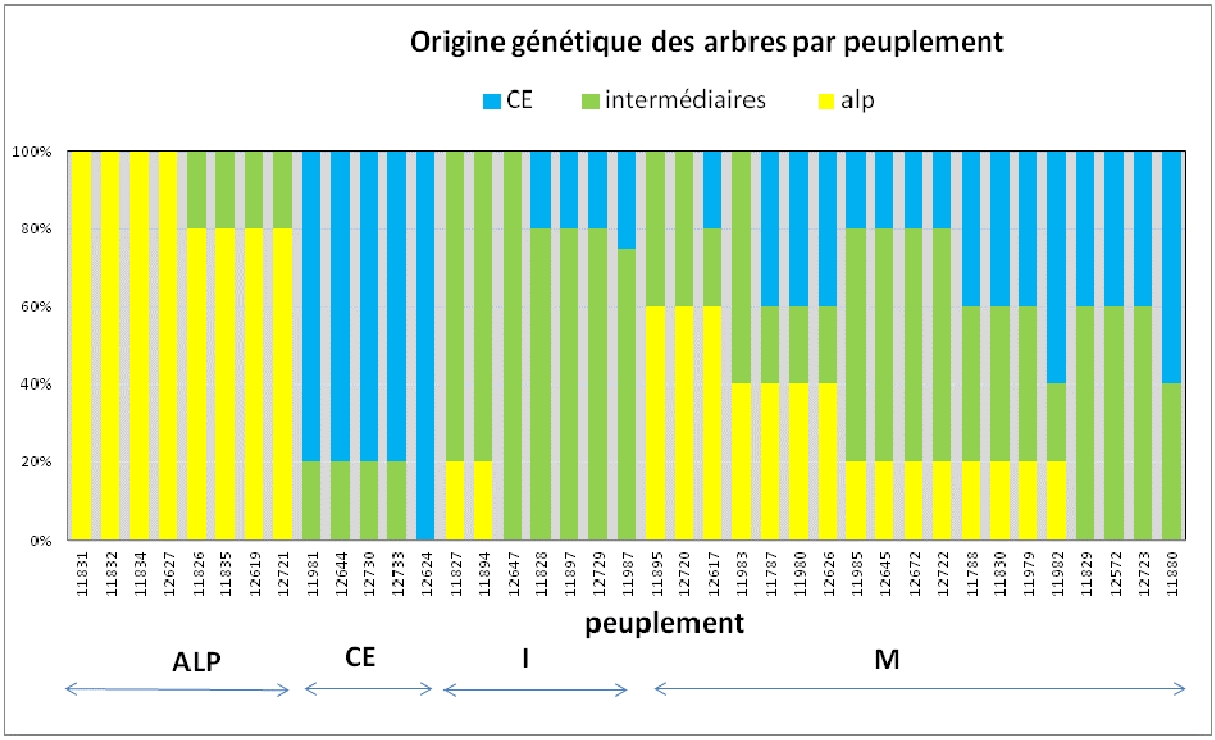

Figure 4. Classification des peuplements en : peuplement d'origine Alpes (ALP), d'origine Europe centrale (CE), peuplement intermédiaire (I) ou mélangé (M) en fonction de la l'origine génétique des arbres étudiés (ALP, CE ou intermédiaire).

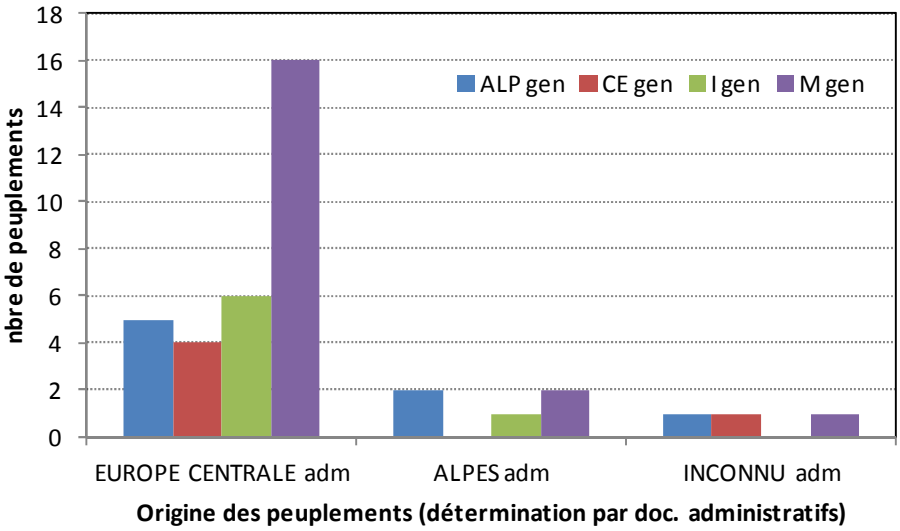

Figure 5 : Classification croisée de l'origine des 39 peuplements de mélèze, en fonction des informations déclarées sur les documents administratifs et des marqueurs génétiques

Impact du chancre du mélèze en fonction de l'assignation génétique des peuplements

Sur la base des documents administratifs, l'enquête DSF conclue à une prévalence importante du chancre du mélèze dans les peuplements d'origine alpine et dans un certain nombre de peuplements supposés d'Europe centrale. Certains peuplements de cette provenance présentent même des notes de sévérité moyenne assez élevées (Fig.6A).
En considérant l'assignation par marqueurs génétiques (Fig. 6B), les peuplements d'Europe centrale ne présentent plus qu'un très faible taux d'arbres atteints et la sévérité moyenne est très faible. En revanche, les peuplements d'origine alpine ou mélangés peuvent montrer des niveaux d'infection élevés. Des notes de forte sévérité ne sont observées que dans ces deux types de peuplements. Les peuplements intermédiaires présentent un comportement intermédiaire.

Discussion et principales conclusions

La détermination de l'origine génétique des peuplements de mélèze du Massif central par marqueurs aboutit à un classement de sensibilité au chancre conforme à ce qui est connu par ailleurs. Les reboisements provenant d'Europe centrale présentent une sensibilité au chancre nettement plus faible que ceux provenant d'Europe de l'ouest ou des Alpes, ce qui est cohérent avec les résultats de Schober (1985) et avec les tests d'inoculations réalisés par Sylvestre-Guinot et Delatour (1983).

A contrario, en considérant que le chancre du mélèze est un marqueur de provenance, on peut considérer que ces résultats valident également les marqueurs génétiques mis au point par Wagner et al. (2012).

L'approche par marqueurs permet de constater que non seulement le nombre d'arbres provenant effectivement d'Europe centrale est beaucoup plus faible qu'attendu (31% contre 79%), mais aussi que de nombreux peuplements du Massif central sont en réalité des mélanges associant des mélèzes provenant à la fois des Alpes, d'Europe centrale et aussi des individus intermédiaires entre ces deux provenances. Ceci aboutit à une diversité génétique finalement beaucoup plus riche qu'attendu, notamment au niveau intra-peuplement. Cette forte diversité n'a probablement pas été correctement appréciée en n'analysant que 5 individus par peuplement. Pour aboutir à une assignation correcte de chaque peuplement, il conviendrait

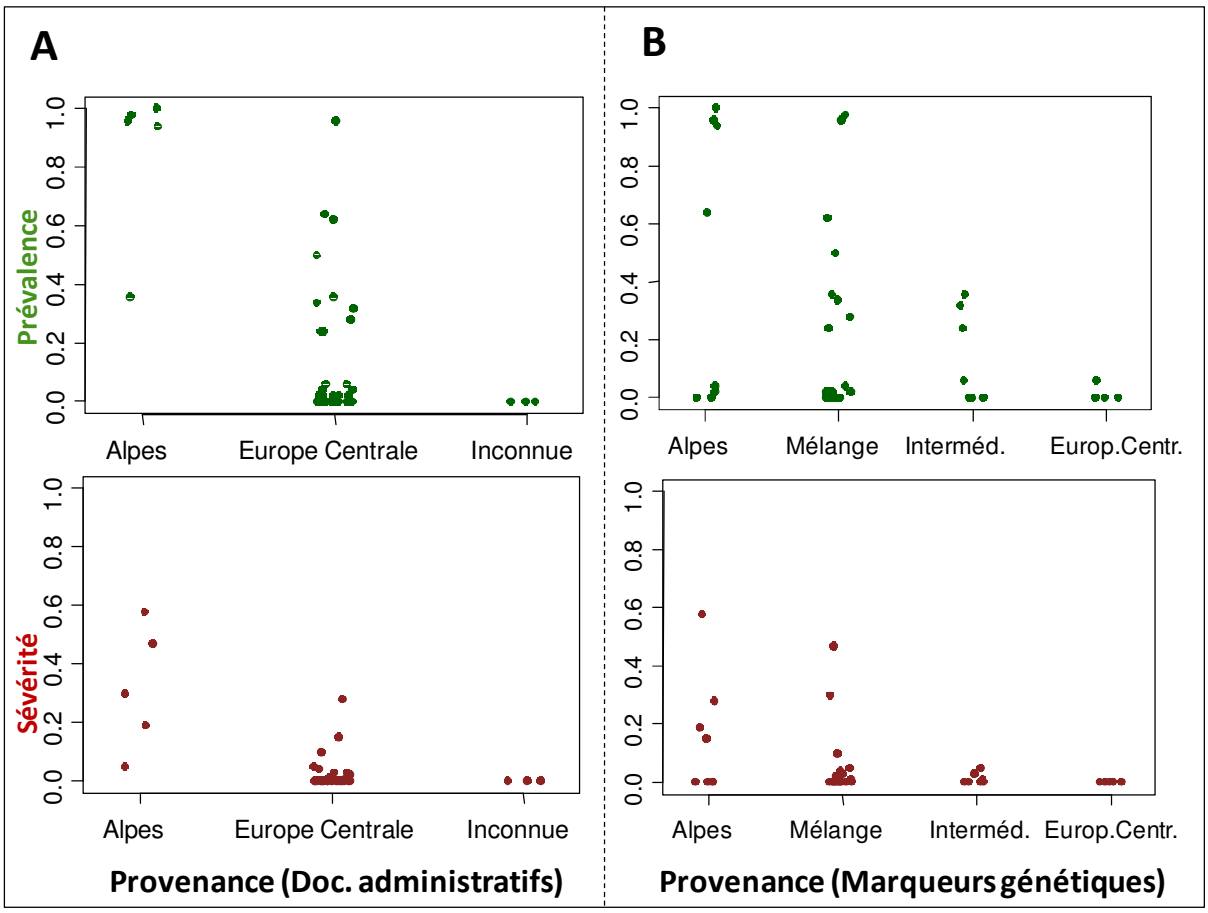

Figure 6 : Prévalence et Sévérité des attaques de chancre dans 39 peuplements de mélèze du Massif central en fonction de l'origine déclarée sur les documents administratifs (A), ou de l'origine déterminée à l'aide des marqueurs génétiques.

éventuellement d'y prélever des échantillons sur 20-30 arbres, ce qui pose ensuite des problèmes de coûts d'analyse.

Cette forte diversité non attendue peut probablement s'expliquer en prenant en compte les bouleversements introduits par l'homme dans les différentes parties de l'aire du mélèze et notamment dans la zone des Sudètes. En analysant plus finement la diversité génétique des peuplements naturels de cette zone, Wagner (in press) a pu démontrer qu'il y avait eu, depuis probablement le 19^{ième} siècle, de très nombreuses introductions de mélèze en provenance d'Allemagne ou du Tyrol autrichien. Il est dès lors vraisemblable qu'une partie des graines récoltées dans des peuplements "naturels" des Sudètes l'ait été en réalité dans des peuplements en grande partie contaminés par des introductions plus ou moins anciennes de mélèzes "alpins" faites à proximité, voire sur des peuplements issus de graines non autochtones.

L'hypothèse de flux naturels de gènes entre le Tyrol et les Sudètes n'est pas à exclure totalement mais ils sont probablement très rares compte tenu de la distance et ils ne pourraient expliquer le nombre particulièrement important d'individus intermédiaires que l'on observe dans notre échantillon. En dernière hypothèse, on peut également penser à des erreurs de manipulation entre la récolte des graines et la vente des plants. Si elles ne sont pas totalement exclues, elles sont cependant peu vraisemblables à une aussi large échelle et n'auraient pas conduit à un aussi grand nombre de peuplements mélangés.

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ZUSAMMENFASSUNG

Diese Dissertation fokussiert die Auswirkungen vergangener Klimaschwankungen und anthropogener Einflüsse auf Populationen der Europäischen Lärche (*Larix decidua* Mill.) mittels Integration paläoökologischer und genetischer Daten. Solche retrospektiven Ansätze liefern eine wertvolle Grundlage für die Vorhersage möglicher Konsequenzen rezenter Entwicklungen. Eine wesentliche Beschränkung existierender Studien an Waldbäumen besteht darin, dass sie häufig ausschließlich die postglaziale Ausbreitungsgeschichte behandeln. Konsequenzen schnellerer Umweltveränderung, einschließlich rezenter Anpflanzungen oder abrupter Klimaereignisse des letzten Glazials auf Wälder, sind weithin vernachlässigt worden. Die vorliegende Studie basiert auf hochauflösenden genetischen Daten und präzisen vegetationsgeschichtlichen Archiven, korreliert mit hochauflösenden Klimarekords des letzten Interglazial/Glazial Zyklus (130 000 Jahre), um kurzzeitige und langzeitige Ereignisse, die die Geschichte der Europäischen Lärche beeinflussten, präzise zu dokumentieren. Für die genetischen Analysen wurden hochinformative nukleare Marker (Mikrosatelliten) entwickelt, die auf ein arealweites Sampling, bestehend aus 45 Populationen, angewendet wurden. Diese nuklearen Daten wurden zusammen mit mitochondrialen Daten analysiert, um eine Grundlage für Studien, die auf die Erforschung rezenter Translokationen zielen, zu schaffen. Die Ergebnisse zeigen, dass *Larix decidua* in erheblichem Maße angepflanzt worden ist, was zur Durchmischung autochthoner und allochthoner Populationen aus multiplen Quellen des Areals führte. Translokationsereignisse und Durchmischungsraten waren ungleichmäßig über das Areal verteilt und besonders häufig in Polen, der Slowakei und Tschechien, wo die Lärche eine verstreutere Verbreitung als in den Alpen aufweist. Einige der wertvollsten Populationen scheinen ernsthaft durch Translokationen bedroht. Die vegetationsgeschichtlichen Befunde zeigen, dass die Lärche den gesamten Interglazial/Glazialzyklus nahe ihres aktuellen Areal überdauerte, jedoch waren die Grenzen der Verbreitung hoch dynamisch und veränderten sich synchron mit kurzzeitigen als auch langzeitigen Klimaschwankungen, was im Einklang mit dem Pioniercharakter der Art steht. Die Verbreitung erreichte ihr maximales Ausmaß zur Zeit des ersten Frühweichsel-Interstadials (87 000 – 109 000 Jahre), als Lärche boreale Wälder im nord-mitteleuropäischen Tiefland bildete. Reaktionen auf Kurzzeit-Klimaereignisse (Dansgaard-Oeschger-Zyklen, Heinrich-Events) waren extrem schnell. Für die Zeit der letzten maximalen Vereisung (LGM) wurden sieben Refugien, basierend auf Fossilien und genetischen Daten, identifiziert. Dies ermöglichte es, Rekolonisierungswege und begleitende Introgressions- und

Homogenisierungprozesse zu ermitteln, was die Leistungsfähigkeit der gekoppelten populationsgenetischen und paläoökologischen Perspektive unterstreicht.

RÉSUMÉ

Dans cette thèse, je m'intéresse aux conséquences sur les populations de mélèze d'Europe (*Larix decidua* Mill) des changements climatiques passés et des changements d'exploitation par l'homme, en intégrant des données paléoécologiques et des données génétiques. Une telle étude rétrospective offre un exemple utile pour évaluer les conséquences possibles des changements actuels. Les études récentes disponibles sur les arbres forestiers sont généralement limitées à l'analyse des recolonisations postglaciaires. Les effets de changements plus rapides observés sur les forêts ont été largement négligés, par exemple les conséquences de plantations récentes ou d'événements climatiques brusques de la dernière période glaciaire. Dans cette étude, j'utilise des données génétiques discriminantes ainsi que des inventaires précis de végétation liés à des relevés climatiques de haute résolution du derniers cycle interglaciaire/glaciaire (130 000 ans), afin d'analyser de façon détaillée les événements récents et plus anciens qui ont affecté l'histoire du mélèze d'Europe. Pour l'analyse génétique, j'ai mis au point des microsatellites, marqueurs génétiques informatifs, avec lesquels j'ai analysé un échantillon de 45 populations provenant de l'ensemble de l'aire de répartition actuelle du mélèze. J'ai analysé ces données de polymorphisme génétique nucléaire en même temps que des données de la diversité mitochondriale afin d'établir des cartes de référence de la diversité génétique naturelle me permettant de détecter des translocations récentes. Les résultats montrent que le mélèze a été planté de façon importante, ce qui a créé des mélanges entre des populations locales et d'autres introduites à partir de sources variées de l'ensemble de l'aire de répartition. Les événements de translocation et les taux de mélange sont répartis de façon hétérogène dans l'aire de répartition, avec une fréquence particulièrement élevée en Pologne, en Slovaquie et au Tchéquie, où le mélèze possède une répartition plus dispersée que dans les Alpes. Quelques-unes des populations de mélèzes présentant un intérêt écologique et économique majeur apparaissent sérieusement menacées par les translocations. Les résultats paléontologiques montrent que le mélèze est resté à proximité de son aire de répartition actuelle pendant le dernier cycle interglaciaires/glaciaires, mais que sa répartition s'est maintenue dans un équilibre dynamique avec les événements climatiques anciens mais aussi avec ceux plus récents, ce qui s'explique

par les caractéristiques pionnières de l'espèce. L'amplitude de répartition de l'espèce a été maximale pendant le premier interstade Weichsélien (87 000 – 109 000 ans) quand le mélèze a contribué à établir les forêts boréales des plaines européennes du nord et du centre. Les réponses aux événements climatiques brefs (*événements Dansgaard-Oeschger et Heinrich*) ont été extrêmement rapides. Sept refuges correspondant aux derniers maximums glaciaires ont été identifiés en utilisant les données fossiles et génétiques. Notre approche nous a permis d'identifier des chemins de recolonisation et les introgressions et homogénéisations concomitantes, illustrant la puissance de l'approche consistant à associer la génétique des populations et la paléoécologie.