New Phytologist

Tree endurance on the Tibetan Plateau marks the world's highest known tree line of the Last Glacial Maximum

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Received: *28 April 2009*
Accepted: *12 July 2009*

New Phytologist (2010) **185**: 332–342
doi: 10.1111/j.1469-8137.2009.03007.x

Key words: glacial refugia, high-mountain areas, *Juniperus tibetica* complex, orographic leading edge, orographic rear edge, Tibetan Plateau.

Summary

• Because of heterogeneous topographies, high-mountain areas could harbor a significant pool of cryptic forest refugia (glacial microrefugia unrecognized by palaeodata), which, as a result of poor accessibility, have been largely overlooked. The juniper forests of the southern Tibetan Plateau, with one of the highest tree lines worldwide, are ideal for assessing the potential of high-mountain areas to harbor glacial refugia.

• Genetic evidence for Last Glacial Maximum (LGM) endurance of these microrefugia is presented using paternally inherited chloroplast markers. Five-hundred and ninety individuals from 102 populations of the *Juniperus tibetica* complex were sequenced at three polymorphic chloroplast regions.

• Significant interpopulation differentiation and phylogeographic structure were detected (G_{ST} = 0.49, N_{ST} = 0.72, $N_{ST} > G_{ST}$, $P < 0.01$), indicating limited among-population gene flow. Of 62 haplotypes recovered, 40 were restricted to single populations. These private haplotypes and overall degrees of diversity were evenly spread among plateau and edge populations, strongly supporting the existence of LGM microrefugia throughout the present distribution range, partly well above 3500 m.

• These results mark the highest LGM tree lines known, illustrating the potential significance of high-mountain areas for glacial refugia. Furthermore, as the close vicinity of orographic rear-edge and leading-edge populations potentially allows gene flow, surviving populations could preserve the complete spectrum of rear-edge and leading-edge adaptations.

Introduction

Based on pollen records, it is generally accepted that temperate and boreal forests of the world alternately became extinct at their poleward edges and expanded from their cold-stage macrorefugia following the climatic oscillations of the Pleistocene (Huntley & Birks, 1983; Bennett *et al.*, 1991; Lang, 1994; Hewitt, 1999; Prentice & Jolly, 2000; Frenzel *et al.*, 2003). However, macrofossil analyses, as well as genetic studies worldwide, are raising doubts about these glacial 'tabula rasa' scenarios for the leading edges (term 'leading edge' used *sensu* Hewitt, 2000). They indicate that pollen records are prone to 'overlook' cryptic refugia (from here on called microrefugia *sensu* Rull, 2009) of small sizes and of very local distribution (Willis *et al.*, 2000; Stewart & Lister, 2001; Willis & van Andel, 2004; Anderson *et al.*, 2006; Shepherd *et al.*, 2007; Pruett & Winker, 2008; Birks & Willis, 2009). Despite this growing evidence, the importance of microrefugia for preserving genetic diversity and speeding up Holocene recolonization is still widely

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underappreciated and interpretations of genetic data too often follow the dictate of pollen data, even when the coverage of pollen records is limited (Provan & Bennett, 2008). This is especially true for high-mountain areas that, because of their heterogeneous topography, should be ideal places for refugia and *in situ* persistence (Hewitt, 2004; Hampe & Petit, 2005) but owing to a lack of sufficient palaeorecords are often overlooked in this context. To demonstrate the potential importance of high-mountain areas for glacial forest endurances we present this case study of the forest history of the southern Tibetan Plateau using genetic markers to test for two possible hypotheses: glacial extinction with postglacial recolonization vs endurance of fragments of an interstadial forest. Both scenarios should provide distinctive genetic patterns: low degrees of genetic diversity within and between populations in the case of a recent recolonization from the plateau edges; and low within-population diversity but high diversity between populations as a result of genetic drift under *in situ* survival.

The Tibetan Plateau is the highest mountain plateau on earth, with 1.9 million km^2 being higher than 4 km above sea level (asl). It is largely covered by alpine pastures and alpine desert-steppe. Forests are limited to the eastern and southern declivities, with only some forest islands occurring on the plateau platform. Most forest stands are composed of *Juniperus*, *Betula* and *Picea* in the northeast (Qinghai province) and of *Juniperus* and *Betula* in the southern part of the Tibetan Plateau. Charcoals and macrofossils indicate a historically larger range of the juniper forests (Kaiser *et al.*, 2006, 2007, 2009; Miehe *et al.*, 2006, 2008). These forest remains have been interpreted as signs of a fragmented Holocene forest belt marginalized by anthropogenic influence as well as desiccation since the mid-Holocene climatic optimum (Miehe *et al.*, 2008; Kaiser *et al.*, 2009). On the basis of the few pollen records available, the existence of these forest islands has been attributed to Holocene recolonizations out of eastern and southeastern lowland refugia (Tang & Shen, 1996).

For the northeastern part of the Tibetan Plateau, this hypothesis has been supported by a phylogeographic study using chloroplast DNA (cpDNA) markers to analyze the genetic structure of *Juniperus przewalskii* forest islands. Six haplotypes were detected whose spatial distribution supports a Holocene recolonization from the eastern declivity (Zhang *et al.*, 2005). Also in the region, a similar pattern was shown for *Picea crassifolia* (Meng *et al.*, 2007).

In the present study, the analysis of forest history was extended to the southern part of the Tibetan Plateau by surveying the historical population dynamics of juniper forests. For this region, Frenzel *et al.* (2003) propose several glacial forest refugia in the deep valley gorges of the Mekong, Salween, Yangtze and Huang He (Fig. 1a). Because of the limited pollen data they draw support from indirect measures linking observed cold and drought limits of the present day's juniper forest distribution with paleoclimatological reconstructions. Based on these reconstructions they suggest an upper tree line between 3450 and 3600 m asl during the Last Glacial Maximum (LGM) (Frenzel *et al.*, 2003). Meanwhile, new records of extant juniper forests were found on the Tibetan Plateau at 4900 m asl, 200 m above the formerly known tree line (Miehe *et al.*, 2007). This extends the known limits of cold tolerance in this species. Following Frenzel's line of argument (Frenzel *et al.*, 2003), the potential LGM upper tree line would increase by 200 m to 3650–3800 m asl and therefore the area of potential LGM forest would increase significantly. Accordingly, and given that the idea of a large Tibetan Plateau ice shield has been soundly rejected (see Seong *et al.*, 2008), large parts of the southern Tibetan Plateau along the Yarlung Zhangbo catchment and the Kyi Chu River up to the city of Lhasa could have potentially supported forest growth during the LGM, thus including valleys throughout the present entire distribution range.

There are two main difficulties encountered when attempting to perform a phylogeographic study of the juniper forests in this region. First, the possibility of obtaining samples is limited because of the remoteness of the few remaining juniper stands. Second, these stands are made up of several tree species whose taxonomic classification is difficult and often arbitrary (Adams, 2004; Farjon, 2005). A morphological screening of several thousand specimens from these species covering their entire distribution range points to incomplete lineage sorting, or massive hybridization with species-specific characters unclearly delimited (L. Opgenoorth, G. Miehe & J. Liu, unpublished). Initial tests based on nuclear microsattelites (SSRs). SSRs also suggest strong hybridization (L. Opgenoorth, unpublished). Because focusing on a single species was thus infeasible, we decided to study a whole complex of closely related, interbreeding tree species, including *Juniperus tibetica*, *Juniperus indica*, *Juniperus convallium*, *Juniperus microsperma* and *Juniperus saltuaria* (Farjon, 2005). According to nuclear ITS Internal transcribed spacer and cpDNA sequences, this group (referred to as the *J. tibetica* complex hereafter) is monophyletic (Adams *et al.*, 2008). As the main focus of this study was not the analysis of introgression and hybridization processes, but the analysis of the large-scale spatial genetic structure of this group of species, we confined the analysis to uniparentally inherited cpDNA markers for inferring glacial refugia. More specifically, we wanted to: establish chloroplast genetic lineages within this complex; outline the phylogeographic and demographic history of each genetic lineage by means of genetic structure and allele frequencies; test the hypothesis of LGM forest endurance on the Tibetan Plateau based on genetic signatures; and integrate the molecular results with other palaeoecological evidence.

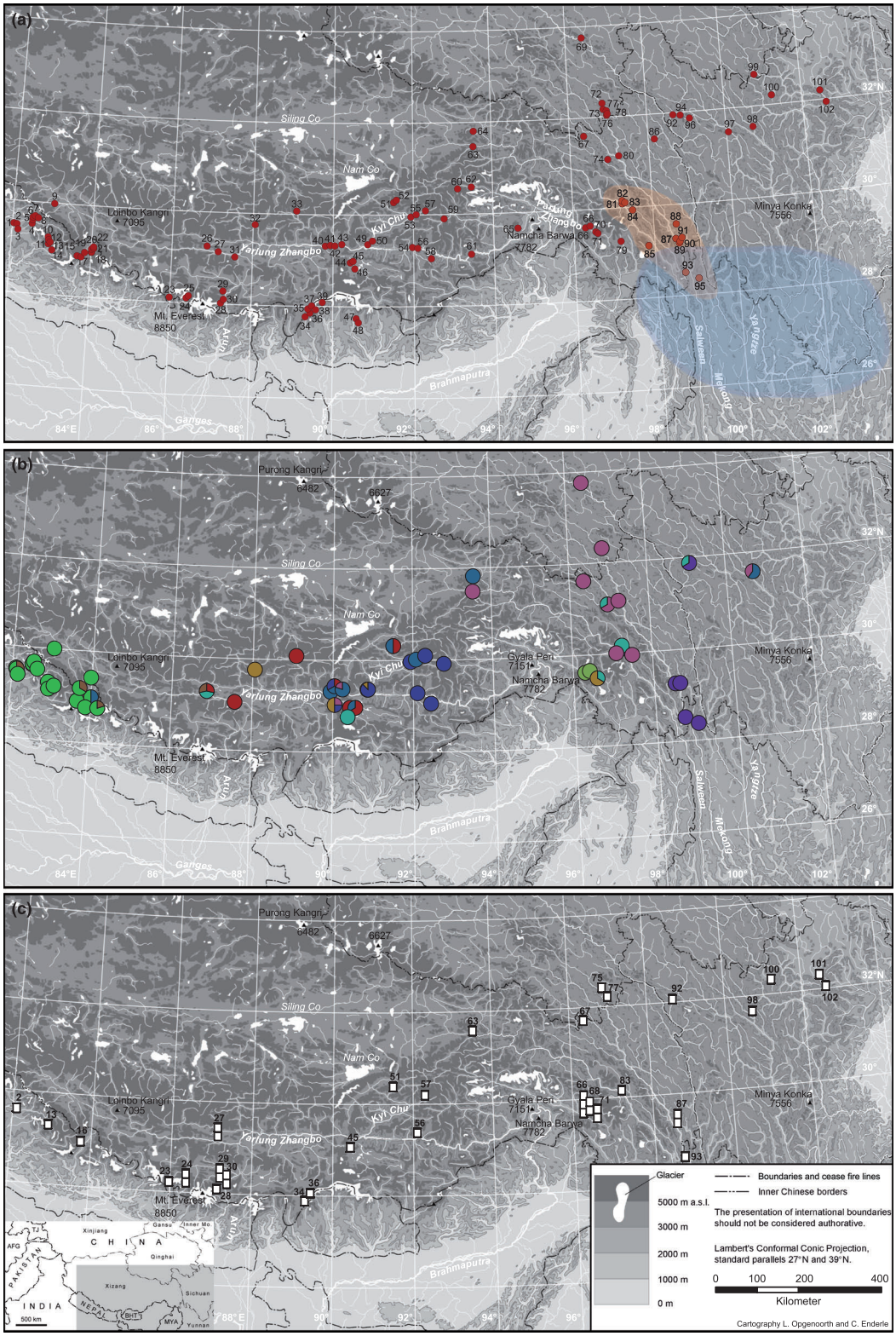

Fig. 1 (a) Map of 102 juniper populations. Orange shading reflects refugia proposed in Frenzel *et al.* (2003). Blue shading reflects a general southeastern refugium proposed by Tang & Shen (1996). (b) Regional distribution pattern of medium frequent haplotypes (8–100 individuals per haplotype). Each color reflects one haplotype. (c) Distribution of private haplotypes. Each column refers to one population. Each box in a column refers to a single private haplotype.

Materials and Methods

According to previous studies on other Cupressaceae species (Neale *et al.*, 1989, 1991; Mogensen, 1996; Kondo *et al.*, 1998; Hwang *et al.*, 2003), both cpDNA and mitochondrial DNA (mtDNA) are generally paternally inherited in this family. We therefore restricted our study of organelle DNA variation to the more polymorphic cpDNA (Petit & Vendramin, 2007). To identify potential cpDNA markers associated with glacial refugia, we first sequenced several noncoding cpDNA regions on a subset of the samples. This initial screening revealed three regions displaying sufficient polyDnaSPmorphism: the *trn*T-*trn*L Intergenic spacer IGS; the *trn*L-*trn*F IGS; and the *trn*L intron. Because these three regions are linked as a result of the uniparental inheritance of chloroplasts, they were combined to derive haplotypes. In total, 102 populations with a total of 590 specimens were sampled within the *J. tibetica* complex with five closely related putative species, namely *J. convallium*, *J. indica*, *J. microsperma*, *J. saltuaria* and *J. tibetica*. The sampled populations covered the whole distribution range of this hybrid complex (Fig. 1a; Table S1). Initial screening of 15 populations with > 10 individuals each showed that an increase in population number, rather than of individuals within populations, allowed detection of the highest haplotypic variation. Thus, for the remaining populations, five to nine samples were taken when available. For 46 sites, only one to four individuals were available because the samples were taken either from herbarium material (most of the Himalayan populations) or from populations of very limited size (one or a few individuals). The populations with one or two individuals were excluded from the statistical analyses.

Total DNA was extracted from leaves, as described by Dumolin *et al.* (1995). PCRs were performed using the primers and protocols described by Taberlet *et al.* (1991). Sequencing reactions were performed using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Munich, Germany) and run on a 96-capillary automated sequencer (MegaBACE 1000; GE Healthcare) following the manufacturer's protocols. Chromatograms were checked using the CHROMAS software (TECHNELYSIUM Pty Ltd, Tewantin, Qld, AU) and sequences were manually edited using CODONCODE v. 1.6.3 (CodonCode Corporation, 2007). Sequences were aligned using the CLUSTAL_X1.83 algorithm (Thompson *et al.*, 1994), as incorporated into CodonCode v. 1.6.3 (CodonCode Corporation, 2007), and corrected manually. They were assigned to different haplotypes using DnaSP 4.20 (Rozas *et al.*, 2003).

Genetic diversity and phylogeographic structure

Different glacial histories have been reported to cause varying patterns of genetic diversity within species. Usually, refugial populations show higher genetic diversity than postglacially established populations, except for situations where several recolonization lineages merge in a postglacially recolonized area (Hewitt, 2000; Petit *et al.*, 2003). However, as merging lineages blur phylogeographic structure, the latter case can be precluded in the event that phylogeographic structure is detected. In order to assess these scenarios, various measures of genetic diversity were estimated and the presence of a phylogeographic structure was tested.

First, allelic richness was estimated, after rarefaction for each population containing more than five samples, using Contrib 1.01 (Petit *et al.*, 1998). In order to test for decreasing diversity along a potential recolonization route, a Spearman rank correlation test between genetic diversity (theta pi obtained with Arlequin 3.11 Schneider *et al.*, 2000) and longitude, and between nucleotide diversity (π, following Tajima 1983, and Nei 1987, as implemented in Arlequin 3.11) and longitude was performed among plateau populations.

The existence of a phylogeographic structure was tested following Pons & Petit (1995, 1996) by calculating two measures of genetic differentiation: G_{ST} and N_{ST}. While G_{ST} is a differentiation measure that is based on allele frequencies only, N_{ST} takes into account the similarities between haplotypes (i.e. the number of mutations between haplotypes). Thus, while high G_{ST} values indicate a general geographic structure in the data, higher N_{ST} values than of G_{ST} values indicate phylogeographic structure, with closely related haplotypes being more likely to co-occur close to each other. Therefore, the two parameters were compared using a permutation test with 10 000 permutations and the *U*-statistic, as implemented in PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/Permut).

Phylogenetic relationships among haplotypes were inferred with MRBAYES 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using an F81+I+G substitution model following Mr.Modeltest2.2 (Nylander, 2004). Indels were treated as a separate block, using p-distances after simple coding (Simmons & Ochoterena, 2000) as implemented in seqstate v.1.32 (Müller, 2005). Bayesian analyses were conducted using two independent Markov Chain Monte Carlo runs with 10 million generations each, sampling trees every 1000 generations and with a burn-in of 1 million generations. One additional specimen (*Juniperus communis*, accession no. GQ301207) was included as the outgroup.

The presence of genetic barriers among populations was tested using the Monmonier maximum-differences algorithm implemented in the BARRIER 2.2 software (Manni *et al.*, 2004). The strength of this method is that it identifies geographic boundaries of abrupt change in genetic differences between pairs of populations based on a network obtained by Delaunay triangulation. Thus, population genetic structure can be analyzed spatially. As this approach requires a priori definition of the number (*K*) of groups, we

ran BARRIER successively on our data set with increasing K until group structures started to dissolve to single populations. The genetic differentiation matrix was obtained using MEGA (Tamura *et al.*, 2007) and was based on p-distances to be able to include indel information.

Inference of demographic processes

Fu's *F*s, as implemented in Arlequin 3.11 (Schneider *et al.*, 2000), and Fu and Li's D^* and F^* statistics, as implemented in DnaSP 4.50 (Rozas *et al.*, 2003), were used to test for deviations from neutrality.

In an attempt to further infer demographic processes, a mismatch distribution analysis was carried out using Arlequin 3.11 (Schneider *et al.*, 2000). The shape of the graph of the mismatch distribution is expected to be multimodal in samples drawn from populations at demographic equilibrium, whereas unimodal distributions are generally found in populations that have passed through a recent demographic expansion (Rogers & Harpending, 1992; Harpending *et al.*, 1998). It is important to stress that the shape of the distribution could potentially also be generated by a bottleneck event, and the distinction between the two demographic processes is difficult (Rogers & Harpending, 1992). The fit of the mismatch distribution to Poisson distributions was assessed by Monte Carlo simulations of 1000 random samples. The sum of squared deviations (SSDs) and raggedness (r) indices between observed and expected mismatch distributions were used as test statistics; their P-values represented the probability of obtaining a simulated SSD that was larger than or equal to the observed SSD.

Finally, separate haplotype networks for each genetic lineage, revealed by the phylogenetic analysis, were constructed using TCS 1.21 (Clement *et al.*, 2000) to obtain additional information about past demographic history of the species. Network ambiguities were resolved following the criteria suggested by Crandall & Templeton (1993): (1) rare haplotypes are more likely to be found at the tip, and common haplotypes found at interior nodes of a cladogram; (2) a singleton (i.e. a haplotype represented by a single individual) is more likely to be connected to haplotypes from the same population than to haplotypes from different populations.

Results

Genetic diversity

Sequencing of the *trn*T-*trn*L IGS, the *trn*L-*trn*F IGS and the *trn*L intron resolved 28 single nucleotide polymorphisms and 16 indels with lengths varying from 1 to 82 bp. These resulted in 62 different haplotypes with sizes ranging from 922 to 1014 bp and an alignment length of 1109 bp (accession no.: GQ268173–GQ268222, GQ285817–GQ285847).

Haplotype frequencies are reported in Table S2. Most of the haplotypes were rare haplotypes with 33 singletons and five haplotypes recorded only twice. The two most frequent haplotypes (H13, H25) accounted for 45% of the samples, and the six most frequent haplotypes accounted for 71% of the samples. Accordingly, while the majority of haplotypes (40) was fixed in one population (Fig. 1c), the three most frequent haplotypes occurred in populations throughout the plateau. Haplotypes of medium frequency generally showed regional distribution patterns (Fig. 1b).

The number of haplotypes per population ranged between 1 and 6 (allelic richness after standardization to a common size of five, using the rarefaction method, ranged between 1 and 4.5, Table S1). Most populations showed little variation. Of the 73 populations with $n \geq 3$, 16 displayed one haplotype, 27 displayed two haplotypes and 22 displayed three haplotypes. Only two populations (P71 and P30) contained six and five haplotypes, respectively. No clear geographic pattern could be observed in the distribution of the populations displaying the highest variation. This can be seen in Fig. 2a, which shows populations with above- and below-average levels of richness. Likewise, the private haplotypes (haplotypes fixed in that population) were evenly spread all over the distribution range (Fig. 1c).

The Spearman rank correlation test between genetic diversity (theta pi) and longitude and between nucleotide diversity (π) and longitude among plateau populations showed no significant correlation (Spearman's rho (rs) = 0.03, P = 0.185; and rs = 0.03, P = 0.179, respectively).

Phylogeographic structure

A strong signal for phylogeographic structure was found. The N_{ST} (0.72) was significantly larger than the G_{ST} (0.49, $P < 0.01$), demonstrating that gene-flow is low relative to mutation rate. Three genetic lineages with distinct distributions were identified (Fig. 3). Genetic lineage 1 (GL1) included haplotypes H1–H4, H16 and H17. Genetic lineage 2 (GL2) comprised haplotypes H35–H39 and H46–H59. These two genetic lineages were distributed in specific geographic areas (Fig. 2b). Genetic lineage 1 comprised populations 66 and 68 in the Parlung Zhangbo valley (Fig. 2b). Genetic lineage 2 was almost exclusively confined to the Himalayan populations, with the exception of H57 and H55 that occurred also in two plateau populations (P9 and P26, respectively) (Fig. 2b). Furthermore, GL2 was subdivided into two subgroups (called GL2a and GL2b Fig. 3), which correspond to separate geographic regions (Fig. 2b).

Genetic lineage 3 (GL3) included all remaining haplotypes, for example, those having no sufficient posterior probabilities and those consisting of too few haplotypes and

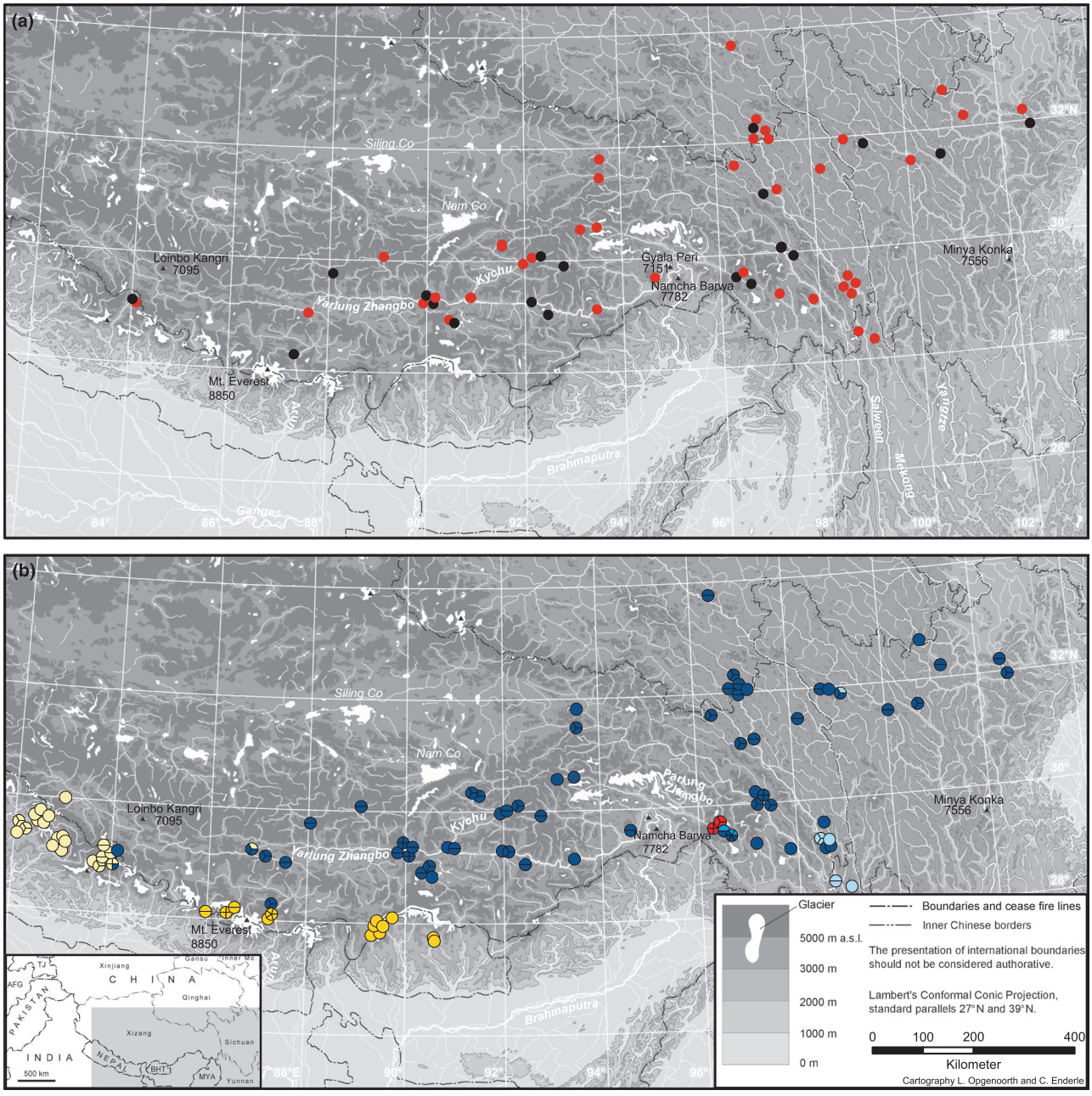

Fig. 2 (a) Juniper populations of above-average (black) and below-average (red) allelic richness. (b) Populations according to genetic lineages (GLs). GL1, red; GL2a, light yellow; GL2b, dark yellow; GL3a, light blue; GL3b, medium blue; GL3c, dark blue. Boxes refer to single haplotypes.

too few splits with just one bifurcation to legitimate separate lineages (H18–H20 and H5–H8; Fig. 3). Nevertheless, both subgroups H18–H20 and H5–H8 are geographically confined to limited areas, thus also contributing to the phylogeographic structure (Fig. 2b).

Two striking disjunctions were observed in GL3, with haplotype H26 occurring in the Himalayan population P20 and in the easternmost plateau population P102, and haplotype H43 was found only in the Himalayan population P17.

Monmonier's maximum difference algorithm confirmed the groups derived by the phylogeny, with a split between the Himalayan populations and the plateau populations, a differentiation of the two Himalayan groups and the split of GL1 from the rest (figure not shown). It did not resolve the split between the GL3 subgroups, however. Additional increase in the number of *K* led to a dissolution of group structure by singling out individual populations with endemic haplotypes.

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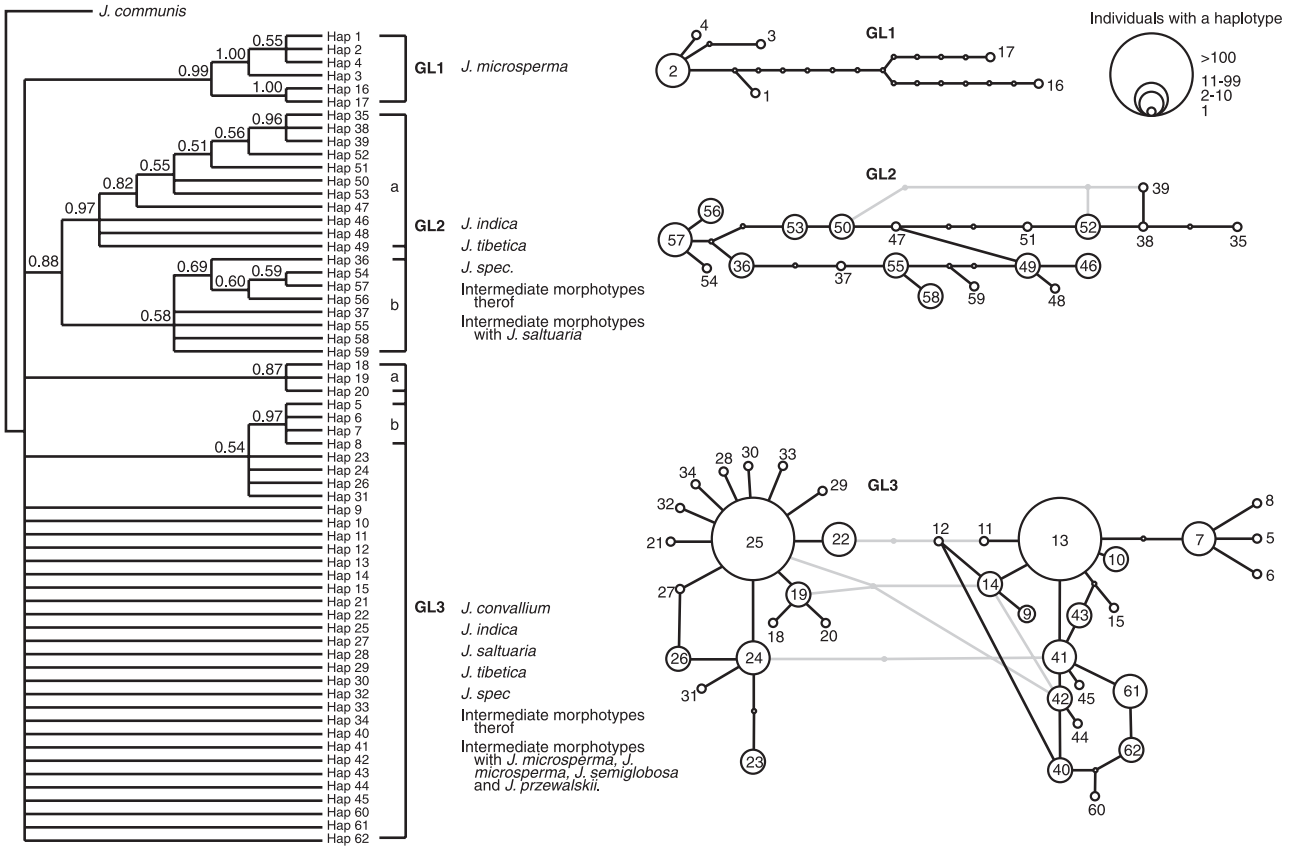

Fig. 3 Phylogeny with genetic lineages (GLs) and putative morphological species with haplotype networks of the respective genetic lineages.

Neutrality tests and demographic processes

Fu's *Fs* statistics produced large negative values for GL2 and GL3, showing deviances from neutrality (Table 1). As Fu and Li's *D* and *F* statistics were both not significant and background selections were thus unlikely causes for the deviance from neutrality, historic population growths or severe bottlenecks remained two possible explanations for the observed patterns (Fu, 1997). For GL1, all neutrality test statistics for deviation from neutrality were nonsignificant (Table 1).

The mismatch distributions for all genetic lineages were unimodal (figures not shown), with the SSD values between the observed and the expected mismatches and Harpending's *r* indices being not significant (Table 1), confirming the neutrality test results for GL2 and GL3 and contradicting the results for GL1.

The minimum spanning network for GL1 showed a star-like pattern (Fig. 3) with one dominating haplotype (H2 comprising 19 of the 24 samples) and five singletons. A star-like pattern is considered to indicate a demographic expansion (Hudson, 1990), thus supporting the findings of the mismatch distribution.

As a result of its complex topography, the GL2 haplotype network gave no clear indication regarding demographic processes (Fig. 3). The uneven sampling in the Himalayan populations could potentially have distorted the frequencies in the minimum spanning network.

Because many haplotypes differed by only one or a few mutations, the GL3 minimum spanning network produced closed loops that could not be resolved in all cases. Nevertheless, the most abundant haplotypes were interior

Table 1 Demographic expansion

GL	SSD	*P*-value	HRI	*P*-value	*Fs*	*P*-value	*D**	*P*-value	*F**	*P*-value
GL1	0.042	0.16	0.348	0.43	0.720	0.67	−1.57	>0.10	−1.97	>0.10
GL2	0.006	0.63	0.018	0.73	−26.0	0.00	−0.61	>0.10	−0.61	>0.10
GL3	0.023	0.06	0.064	0.13	−26.5	0.00	−2.05	>0.05	−1.79	>0.10

*D**, Fu and Li's *D** test statistic; *F**, Fu and Li's *F** test statistic; *Fs*, Fu's *Fs* test statistic; GL, genetic lineage; HRI, Harpending's raggedness index; SSD, sum of squared deviation under expansion model.

haplotypes, and most of the singletons were tip haplotypes (Fig. 3), as would be expected under coalescent theory (Hudson, 1990). The overall network is as complex as the GL2 but it comprised some clades with star-like patterns, hinting at demographic expansions within some of the subclades.

Discussion

The classical glacial 'tabula rasa' scenario for forests on the Tibetan Plateau assumes the complete extinction of the former interstadial forests and postglacial recolonization from southern and eastern macrorefugia located in the deep valley gorges (Tang & Shen, 1996). Accordingly, as observed for *J. przewalskii* in Qinghai (Zhang *et al.*, 2005), all haplotypes recorded at present would have dispersed from these refugia or would have appeared during the potential range expansion. The high amount and even distribution of private haplotypes throughout the range of the *J. tibetica* complex provides a very strong argument against this scenario. As dispersal of private haplotypes to single populations throughout the range is very unlikely, these private haplotypes would have had to evolve within a time span of approx. 14 000 yr or less following the LGM. Even though exact mutation rates are not available for the chloroplast sequences employed in this study, the information available for cpDNA in general, as well as results with the same sequences reported in other tree species (Anderson *et al.*, 2006; Magri *et al.*, 2007), clearly contradict this hypothesis. For example, Graur & Li (2000) report an average mutation rate of $1.2–1.7 \times 10^{-9}$ substitutions per site per year for cpDNA. Given that the average sequence length in this study is 1000 bp, a single mutation in one of those sequences should occur every 580 000–1 000 000 yr. Thus, even if all 62 haplotypes detected in this study would differ by only one mutation to all other haplotypes it would be highly unlikely that all of these mutations occurred within the past 20 000 yr. Instead, there are several haplotypes, even from the western margin of the plateau, that carry two substitutions compared with their closest recorded relative (e.g. H23 and H60), thus increasing the time span to 1 060 000–2 000 000 yr or even longer when considering the closest plateau edge haplotype. Furthermore, if these mutations had occurred during such a short time span, one would expect higher overall haplotypic diversity, in particular in refuge populations, considering that they had at least an order of magnitude more time for haplotype accumulation.

Instead, a large proportion of the populations are fixed for one or two haplotypes regardless of their geographic location. Furthermore, overall degrees of diversity are not significantly different between the plateau populations and the Himalayan populations. This also holds true when comparing the diversity of the plateau populations of this study with that of plateau edge populations for *J. przewalskii* in the northeast (Zhang *et al.*, 2005). Additionally, if all plateau populations had been extirpated during the LGM, successive founder events during the recolonization would have led to a decline of intrapopulation diversity along the recolonization route. This phenomenon has been frequently observed in the European biota along a south–north gradient (Hewitt, 1996; Comps *et al.*, 2001). As we could show that the Himalayan populations did not contribute to the current populations of the plateau, a potential recolonization would have had to follow an east–west route and thus a decline should be recognizable along that route. This could not be found among the juniper populations analyzed in this study.

A corroborative line of argument against the notion of postglacial recolonization relies on the finding of distinct regional geographic patterns observed with haplotypes of intermediate abundance (Fig. 1b). Except for the haplotypes from GL1 that are linked to *J. microsperma*, the distinct regional patterns do not reflect species boundaries but instead seem to be largely independent from the taxonomic identity of the samples. Thus, such a spatial genetic pattern can either be attributed to populations recently founded by long-distance dispersal events during recolonization (leptokurtic dispersal) (Hewitt, 1993; Ibrahim *et al.*, 1996; Bialozyt *et al.*, 2006), or they can be explained by demographic re-expansion of previously present populations that had recently experienced fragmentation accompanied by bottleneck events and genetic drift. The strong phylogeographic structure indicates that gene flow among the Tibetan Plateau junipers has been severely limited, at least since the formation of the observed geographic/genetic pattern. High G_{ST} values could potentially reflect vegetative reproduction, although this phenomenon has never been reported for these species nor has it been observed in any of the > 100 populations screened for this research. Nevertheless, because long-distance dispersal events tend to minimize phylogeographic structure (Petit *et al.*, 2004), they do not seem to have played a significant role in juniper dispersal. This also has implications for interpreting the geographical haplotype disjunctions identified. Such disjunctions can be attributed to homoplasy, long-distance dispersal or to the fragmentation of formerly more widespread haplotypes. As homoplasy is unusual in the conservative chloroplast genome and long-distance dispersal has also been shown to be an unlikely contributor, the presence of these disjunctions also seems to contradict postglacial recolonizations and to suggest that the fragmentation of formerly more widespread haplotypes is involved.

Finally, the strong phylogeographic structure demonstrates that the Himalayan populations generally did not contribute to the current colonization of the plateau, even though they were much closer to the upper Yarlung Zhangbo and the Kyi Chu catchment populations than a

potential southeastern refugium. This pattern does not seem plausible under a postglacial recolonization scenario that is dependent on high postglacial migration rates. Again, a model of ancient fragmented forest patches appears to explain these results more parsimoniously.

The genetic data presented here strongly suggest that the juniper forest islands and isolated tree stands of the southern Tibetan Plateau are remnants of a former interstadial forest that were fragmented during the last LGM and that experienced postglacial local expansions before again experiencing fragmentation and marginalization as a result of anthropogenic influence as well as desiccation. In addition, we speculate that the clear separation of the Himalayan haplotypes reflected a much older haplotype-distribution pattern, possibly dating back to stages of the uplift of the Himalayas and the plateau during the late Tertiary. Similar cases have recently been made for *Quercus suber* in the Mediterranean basin (Hampe & Petit, 2007; Magri *et al.*, 2007) where the pattern was correlated with the break up and separation of several microplates during the Miocene, and for *Quercus lobata* in California, whose genetic structure was found to 'most likely reflect[s] the impact of the Tertiary' (Grivet *et al.*, 2006). Interestingly, the clear split between Himalayan haplotypes and plateau haplotypes did not strictly follow the orographic barriers, as Himalayan haplotypes 'leaked' onto the plateau at the western margin of the Kyi Chu watershed, while plateau haplotypes reached 'Himalayan territory' in the Arun watershed (Fig. 2b), diminishing the role of the mountain range as a migration barrier.

These results clearly contradict the former perception of forest glacial history on the southern Tibetan Plateau and the phylogeographic results reported for *J. przewalskii* on the northeastern Tibetan Plateau (Zhang *et al.*, 2005). The different fate of the plateau platform populations of *J. przewalskii* in the northeast and the species reported on in this study can probably be attributed to the topographical and ecological differences of these regions. Most notably, the intersecting valleys of the large rivers lead to a larger altitudinal amplitude of approx. 400 m in the distribution range of southern plateau platform populations, in contrast to the distribution range of northeastern plateau populations of *J. przewalskii.* This larger altitudinal amplitude resulted in a larger climatic buffer of > 2° (0.55°/100 m Bohner, 2006) for southern tree populations during the LGM. Moreover, while the altitudes increase gradually from edges to platform on the northeastern Tibetan Plateau, the altitudinal amplitudes of the river valleys are contained and thus provide niches throughout the southern Tibetan Plateau, especially considering that relatively steep southerly exposed valley slopes would receive additional solar heat.

As the juniper tree species can be considered keystone species on the Tibetan Plateau, these findings considerably alter the preconditions for understanding the glacial history of other plant and animal species in the region. Likewise, the ongoing discussion on whether humans inhabited the plateau throughout the LGM or whether they recolonized the plateau at the onset of the Holocene (Aldenderfer, 2006) can profit from these findings as even small forests or woodlands could have provided humans with additional essential resources.

On a global scale our findings stress the potential for microrefugia in the patchy landscapes of high-mountain areas with their availability of diverse environments. This is emphasized by the fact that the valley bottoms of the refugia presented here largely exceed 3500 m, and partly even 4000 m, asl and thus mark the highest LGM tree lines known in the world so far. Furthermore, our results stress the importance of small surviving populations for a species' demographic and evolutionary history. This is even more so when considering the adaptive potential of microrefugia in high mountain areas where strong elevational gradients have a similar effect on populations as have leading and stable rear edges ('stable rear edge' *sensu* Hampe & Petit, 2005) in latitudinal ranges by maintaining adaptive potential for heat and drought tolerance at low altitude (or at the stable rear edge) and cold tolerance and dispersal ability at high altitude (or at the leading edge) (Hampe & Petit, 2005). However, by contrast to the latitudinal rear and leading edge populations, orographic rear and leading edges are in close vicinity to each other, potentially allowing for gene flow. We thus propose that refugia in the orographic stable rear edges could even harbor both adaptive potentials, by bridging the gap between leading and rear edges and thus increasing their evolutionary importance.

In conclusion, the ecological and evolutionary significance of high-mountain areas needs serious re-appraisal, both in terms of conservation efforts as well as in understanding the evolutionary history of species. In addition, the ever-increasing number of microrefugia detected provokes re-evaluation of the importance and speed of postglacial migration processes and population dynamics. In turn, prediction on the impact of future climate change on such dynamics will also have to change.

We hope that this study will encourage further studies aiming to identify and analyze microrefugia, especially in high-mountain areas, to scrutinize forest histories and to assess the adaptive potential of such populations.

Acknowledgements

The authors thank the members of the Lhasa University Expedition Team 2004 for assistance with field data collection. Rémy Petit and Martin Lascoux, as well as three anonymous reviewers, provided critical comments on an earlier version of the manuscript. This research was supported by grants from the German Research Council (DFG Grants Mi 271-18 and Zi 698-6), the German Academic Exchange

Program (DAAD) and by grants from the National Natural Science Foundation of China (30430560, 30725004) to J.Q.L. The original manuscript was linguistically improved by Anita Hopes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Population description and their diversity measures

Table S2 Distribution of haplotypes among populations

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