Estimating the timescale of *Lobaria* diversification

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Abstract: Using an ITS mutation rate as calibration reference, a three-locus timetree was generated for the genus *Lobaria* and its most important clades. The timetree resolved most clades with strong support and gave an estimate of the diversification time for *Lobaria* during the early Oligocene. A fossil impression from a 12–24 million-year-old Miocene deposit is hypothesized here to belong to an ancestral *Lobaria* species. Additionally, the age estimate indicates that the paleoclimate and the closing or opening of the Bering Strait played a major role in shaping the current distribution of most *Lobaria* species. It is hypothesized that the Bering land bridge acted as a major highway during warm-temperate climate periods, but as a barrier during Arctic climate times.

Keywords: ascomycetes, Bering land bridge, disjunction, fossil impression, lichens, mutation rate, time-calibrated phylogeny

Introduction

Internally stratified fossils from the Lower Devonian and divergence time studies based on fungal fossils have demonstrated that lichenization is an ancient mode of fungal nutrition (Lücking *et al.* 2009; Honegger *et al.* 2013; Prieto & Wedin 2013; Beimforde *et al.* 2014). However, fossil records of extant lichen species are scarce. Most important discoveries are from amber, including *Anzia* Stitzenb. from 35–40 Myr-old Baltic amber and *Parmelia* Ach. and *Phyllopsora* Müll. Arg. from 15–45 Myr-old Dominican amber, which indicate a minimum age estimate for the family *Parmeliaceae* Zenker of *c.* 40 Myr (Poinar *et al.* 2000; Rikkinen & Poinar 2002, 2008). However, these fossils were probably formed well after the origin of their family since a fossil-calibrated phylogeny has estimated *Parmeliaceae* to have diversified much earlier, around the Cretaceous-Paleogene boundary 58–74 Myr ago (Amo de Paz *et al.* 2011). Fossils of crustose lichens from the genera *Calicium* Pers. and *Chaenotheca* (Th. Fr.) Th. Fr. have

also been found in 35–55 Myr-old Baltic amber (Rikkinen 2003) and a fossil-calibrated phylogeny that includes different alternative positions of this *Calicium* fossil suggests that *Caliciaceae* Chevall. diversified 103–156 Myr ago in the early Cretaceous (Prieto & Wedin 2016). Finally, amber inclusions of a small number of beard lichens have been found representing either *Oropogon* Th. Fr. or some alectorioid genera (Rikkinen & Poinar 2002, 2008; Rikkinen 2003; Kaasalainen *et al.* 2015).

For the lichen family *Lobariaceae* Chevall., only one fossil impression has been found in a 12–24 Myr-old Miocene deposit from northern California (MacGinitie 1937; Peterson 2000). The impression shows a thallus with the lower side facing upwards (Fig. 1, bottom photograph). The overall appearance of this fossil reveals strong similarities to extant species of *Lobaria* (Schreb.) Hoffm., including traits such as a thallus with elongated lobes, reticulated ridges and small pits along the lobe margins that Peterson (2000) interpreted as impressions of broken soralia. With regard to these morphological traits, Peterson (2000) placed emphasis on the resemblance to *L. pulmonaria* (L.) Hoffm. as well as other species of *Lobariaceae* with reticulated ridges. In fact, the fossil impression resembles three

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living species of *Lobaria*. The reticulated ridges and soralia at the lobe margin of the fossil are typical for *L. pulmonaria*. However, *L. anomala* (Brodo & Ahti) T. Sprib. & McCune also forms reticulated ridges and soralia, and *L. retigera* (Bory) Trevis. forms clusters of slender isidia which are difficult to distinguish from soralia if poorly developed or damaged on the fossil impression. These three species all grow along the western Pacific coast where the fossil impression was found. Furthermore, Cornejo & Scheidegger (2015) have shown that *L. anomala* and *L. retigera* belong to a cyanobacterial lineage whereas *L. pulmonaria* belongs to a green-algal lineage, with both lineages originating from a common ancestral *Lobaria* species. Thus, another possibility is that the fossil impression represents an ancestral taxon.

Fossils are frequently ambiguous because convergent evolution among extinct and living lineages is common (Wang & Mao 2016). Therefore, caution should be employed when integrating ambiguous fossils as minimum age constraints, or integrating unambiguous fossils as maximum age constraints into molecular dating. In the present case, the unambiguous phylogenetic assignment of this fossil is difficult. Therefore, the current study uses a molecular clock-calibrated phylogeny to obtain a time estimate for species of *Lobaria* that can be contextualized against the age of the fossil impression. Here we propose the first age estimate for the genus and discuss whether the fossil impression can be assigned to an extant or an ancestral *Lobaria* species.

Material and Methods

For the present investigation we used the alignment from a previous study on evolutionary relationships within *Lobaria* (Cornejo & Scheidegger 2015), which is available in TreeBase (http://treebase.org) under the study identification number S18155. Three molecular markers (EF-1α, nrITS, *RPB2*) and 50 specimens from all major *Lobaria* clades were assembled and four outgroup specimens of *Lobarina scrobiculata* (Scop.) Nyl. completed the dataset. All calculations were run in BEAST v1.8.4 (Drummond & Rambaut 2007; Drummond *et al.* 2012). First, three analyses using an uncorrelated lognormal relaxed clock were performed (each with a constant speciation rate per lineage (Yule speciation) and 12 million generations) to have an indication of how

clock-like the genes behaved over the phylogeny. With branch rate standard deviations (ucld.stdev) of 0·618 for EF-1α, 0·461 for nrITS and 0·715 for *RPB2* (mean of three runs), data were assumed to be quite clock-like (BEAST 1.8 guide; Drummond *et al.* 2007). Thus, a strict clock prior was used for all analyses. This is the basic model in BEAST which assumes a global clock rate with no variation among lineages in a tree.

Regarding several age estimates for genera of the *Parmeliaceae* and *Lecanoraceae*, we used the mutation rate calculated for 1) nrITS ($2·4 \times 10^{-9}$ substitutions per site per year (s s^{-1} y^{-1}); Table 1) and 2) *RPB2* ($1·5 \times 10^{-9}$ s s^{-1} y^{-1}; Table 1) as secondary calibration points for two separate age estimates, and 3) both mutation rates in a combined calibration approach. The clock model was unlinked and divergence times were estimated under a strict molecular clock, implementing a Yule tree prior which assumes a constant speciation rate per lineage. The data matrix was partitioned by individual gene regions with a linked substitution model (HKY+I+Γ) across loci. Tree parameters were linked across partitions and all other priors and operators were allowed to auto-optimize. The analysis was run with 70 million generations, sampling every 2000th generation, with a discarded burn-in of 10%. Convergence and the consequent proportion of burn-in were assessed using Tracer v1.6 (Rambaut *et al.* 2014), considering effective sample size values >200 as good indicators. A maximum clade credibility tree was generated by analyzing the BEAST trees in TreeAnnotator v1.8.4. This program determined the 95% highest posterior densities (HPD) and estimated the mean heights. The best resulting tree was graphically represented in FigTree v1.3.1 (Rambaut 2009) and edited using OmniGraffle graphic software v6.0.5 (OmniGroup, Seattle, USA).

Results and Discussion

Molecular clock rate

All molecular clock analyses returned a topology that was fully consistent with the previous multilocus phylogeny of *Lobaria* (Cornejo & Scheidegger 2015). We first tested nrITS and *RPB2* mutation rate priors separately and then in a combined approach. Both separately estimated posterior times differed substantially, placing the origin of *Lobaria* either at *c.* 27 Myr BP (nrITS; Fig. 1) or at *c.* 15 Myr BP (*RPB2*; see Supplementary Material Fig. S1A, available online). In the latter case, the fossil impression would just match the diversification of *Lobaria*, which is hypothetically possible. However, the parallel estimated mutation rates for both other loci were highly accelerated (EF-1α $= 1·92 \times 10^{-9}$ s s^{-1} y^{-1}; nrITS $= 4·44 \times 10^{-9}$ s s^{-1} y^{-1}) compared with results for

TABLE 1. *Publication list of lichen studies that calculated the mean mutation rates for loci that are relevant to the present study. Dashes indicate missing information.*

Family	Taxon	Mean rate (substitutions site^{-1} y^{-1} × 10^{-9})			Reference
		EF-1a	nrITS	*RPB2*	
Parmeliaceae	*Melanelixia fuliginosa/ M. glabratula* group	–	2·43[*]	1·50[*]	Leavitt *et al.* 2012a
	Melanohalea	–	3·41[**]	1·51[**]	Leavitt *et al.* 2012b
	Oropogon	–	2·40[***]	–	Leavitt *et al.* 2012c
	Xanthoparmelia	–	2·42[†]	1·42[†]	Leavitt *et al.* 2013a
Lecanoraceae	*Rhizoplaca melanophthalma* s. lat.	1·19[†]	2·41[†]	1·26[†]	Leavitt *et al.* 2013b
	Rhizoplaca	0·98[‡]	2·43[‡]	1·47[‡]	Leavitt *et al.* 2016

[*] Derived from the mutation rates of *RPB1* and nrLSU for *Melanelixia* (Amo de Paz *et al.* 2011).
[**] Derived from the mutation rates of *RPB1* for *Melanohalea*, as well as nrLSU and mrSSU for *Parmeliaceae* (Amo de Paz *et al.* 2011).
[***] Derived from the mutation rate of nrITS for *Melanohalea* (Leavitt *et al.* 2012b).
[†] Derived from the mutation rate of nrITS for *Melanelixia* (Leavitt *et al.* 2012a).
[‡] Derived from the mutation rate of nrITS for *M. fuliginosa/M. glabratula* group (Leavitt *et al.* 2012a).

other lichen taxa (Table 1) and appeared less plausible for the slow-growing *Lobaria* species with rather long generation cycles (e.g. 10 years in *L. pulmonaria*; Scheidegger 1995).

From a statistical point of view, age estimates based on a single calibration are more prone to error than those in which multiple calibrations are applied. One approach to mitigate this error is to apply a combination of two secondary calibrations or, alternatively, of primary and secondary calibrations. Analysis 3, based on both mutation rates in a combined calculation, resulted in mean age estimates that are between both other analyses and placed the origin of *Lobaria* at 20·2 Myr (Supplementary Material Fig. S1B). However, it should be noted that dos Reis & Yang (2013) and Schenk (2016) demonstrated that narrower minimum and maximum age estimates are accounted for by conflicting calibration references rather than more realistic divergence times. We thus assumed that the nrITS-calibrated analysis was more reliable than the *RPB2* or combined estimate. The timetree obtained from analysis 1 is discussed below (Fig. 1), while the *RPB2* and combined calibration trees may be consulted as online supplementary material (Fig. S1).
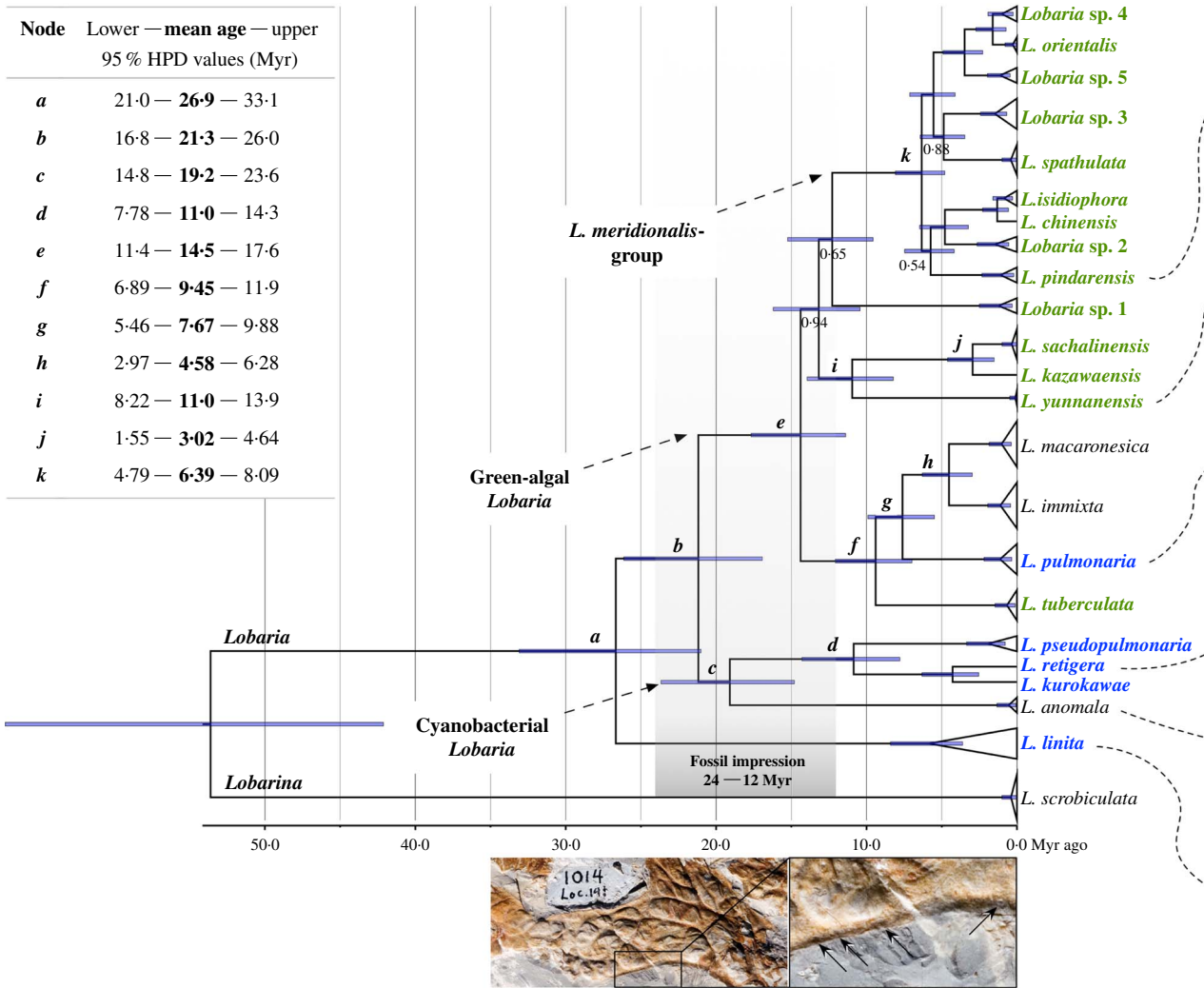
Along with the mutation rate for the nrITS (2·4 × 10^{-9} s s^{-1} y^{-1}), we obtained rates for *RPB2* (0·882 × 10^{-9} s s^{-1} y^{-1}) and EF-1α (1·09 × 10^{-9} s s^{-1} y^{-1}). Most bifurcations are maximally supported by posterior probabilities (PP) = 0·98, except for four that were also poorly supported in the prior analysis (Cornejo & Scheidegger 2015) in which relationships between species of *Lobaria* were discussed. Consequently, these bifurcations are not considered for the age estimate. The following deals exclusively with the age estimate shown in Fig. 1.

Age estimates for *Lobaria*

The most recent common ancestor for species of *Lobaria* was dated to 26·9 Myr BP (node ***a***). Similar ages are known for some genera in the family *Parmeliaceae*, such as *Flavoparmelia* Hale and *Xanthoparmelia* (Vain.) Hale (Amo de Paz *et al.* 2011), *Montanelia* Divakar *et al.* (Divakar *et al.* 2012) and *Melanohalea* O. Blanco *et al.* (Leavitt *et al.* 2012a). Our analysis suggests that several major divergence events led to the evolution of extant taxa within *Lobaria*. In the early Miocene, an ancestral *Lobaria* split into two major lineages, one associated with cyanobacteria (*Nostoc* spp.) and the other comprising several lineages associated with green-algal strains of the genus *Symbiochloris* (*Trebouxiaceae*) (Škaloud *et al.* 2016). Node ***c***

Node	Lower — **mean age** — upper 95 % HPD values (Myr)
a	21·0 — **26·9** — 33·1
b	16·8 — **21·3** — 26·0
c	14·8 — **19·2** — 23·6
d	7·78 — **11·0** — 14·3
e	11·4 — **14·5** — 17·6
f	6·89 — **9·45** — 11·9
g	5·46 — **7·67** — 9·88
h	2·97 — **4·58** — 6·28
i	8·22 — **11·0** — 13·9
j	1·55 — **3·02** — 4·64
k	4·79 — **6·39** — 8·09

L. meridionalis- group

Green-algal *Lobaria*

Lobaria

Cyanobacterial *Lobaria*

Lobarina

Fossil impression 24 — 12 Myr

Lobaria sp. 4
L. orientalis
Lobaria sp. 5
Lobaria sp. 3
k 0·88
L. spathulata
L. isidiophora
L. chinensis
Lobaria sp. 2
0·65
0·54
L. pindarensis
Lobaria sp. 1
0·94
j *L. sachalinensis*
i *L. kazawaensis*
L. yunnanensis
e
h L. macaronesica
g L. immixta
b *f* *L. pulmonaria*
L. tuberculata
a *d* *L. pseudopulmonaria*
c *L. retigera*
L. kurokawae
L. anomala
L. linita
L. scrobiculata

50·0   40·0   30·0   20·0   10·0   0·0 Myr ago

1014
Loc.19

diversified 19·2 Myr BP early in the Miocene and contains four species in this analysis although, taking the missing species-pairs in our dataset into consideration, this clade contains six taxa. The basal radiation of the green-algal species was estimated to have taken place in the middle Miocene (node *e*, 14·5 Myr BP). The disjunct segregation of south- and north-eastern Asian lineages is noticeable in clade *i*. *Lobaria yunnanensis* Yoshim. is known from the mountainous Yunnan Province and Taiwan, while the species pair *L. sachalinensis* Asah. – *L. kazawaensis* (Asah.) Yoshim. grows in deciduous forests in north-eastern Asia. Node *k*, including species of the *L. meridionalis*-group, began diversifying in the late Miocene 6·39 Myr BP. This clade, which includes species exclusively from East Asia that are taxonomically still under dispute, will be discussed in detail elsewhere (C. Cornejo, S. Chabanenko & C. Schei-degger, unpublished data). Node *f* (9·45 Myr BP) includes the widely distributed *L. pulmonaria*, and *L. tuberculata* Yoshim. which is found in a restricted area of temperate and boreal forests in northern Japan, the Russian Primorsky Krai, and in the Sakhalin Oblast' region. Also included are *L. macaronesica* Cornejo & Scheid. and *L. immixta* Vain. (node *h*, 4·58 Myr). These occur only in subtropical laurisilva habitats in the Macaronesian Islands.

These age estimates are somewhat limited because we used a secondary calibration reference derived from the family *Parmeliaceae*. In addition, we cannot be certain that there is a linear relationship between distance and time of *Parmeliaceae* and *Lobariaceae* because molecular clock rates can vary greatly among genes and organisms (Ayala 1997; Takahata 2007; Wilke *et al.* 2009). More widely used approaches for estimating mutation rates involve fossil and/or biogeographical data. However, fossils can only provide minimum age estimates given that a fossil is necessarily younger than the phylogenetic event that led to its existence (Wilke *et al.* 2009). In the present study, the *Lobaria* fossil cannot be assigned unambiguously to branches in a phylogeny because it is possible that the fossil is an extinct taxon.

Another issue is the under-sampling problem. In many cases we do not know whether a phylogeny is complete or not because fossil evidence of extinct lichen species is extremely rare. Furthermore, the interpretation of the phylogenetic relevance of divergence events may be affected by missing taxa and thus subject to misinterpretation. With regard to this question, Cummings & Meyer (2005) point out that sampling manifested through the processes of speciation will tend to lead to short branches and the processes of extinction will tend to lead to longer branches in a phylogeny. For example, the single branching of *L. anomala* in node *c* and the very old age estimate of *c*. 19 Myr for this species suggest an under-sampling problem in our dataset. It is conceivable that taxa became extinct between nodes *c* and *d*. If this were the case, node *c* would represent the maximum age of cyanobacterial *Lobaria* species. Missing taxa could not lead to a greater age calculation for cyanobacterial *Lobaria*, but could lead to a lower age for *L. anomala*. The situation is similar for *L. yunnanensis* and *Lobaria* sp. 1, whose long branches indicate missing taxa. Both species are from understudied regions in East Asia and, in this case, it is also possible that missing taxa are undescribed but presently living species.

FIG. 1. Timetree of the genus *Lobaria* with images of modern *Lobaria* spp. (right) together with the fossil impression (bottom middle) for comparison. The timetree was obtained using Bayesian analysis with the mutation rate of nrITS as calibration reference. All branches obtained posterior probability values ≥0·98, except four with lower values indicated below the branches. Fossil impression age indicated by grey area. Mean ages of nodes *a–k* are given in the table (top left). Species in green are exclusively from East Asia, those in blue also grow in North America and other regions, leaving only *L. anomala* (West Pacific coast), *L. immixta* and *L. macaronesica* (both Macaronesian Islands in the North Atlantic) which grow outside of East Asia. Overview of the fossil impression UCMP No. 1014 (bottom middle left) and an enlargement of the area in the rectangle (bottom middle right). Arrows indicate small pits along the lobe margin (photograph courtesy of E. B. Peterson). *Lobarina scrobiculata* used as outgroup.

Fossil impression: living or extant taxon?

The tree topology in Fig. 1 shows the placement of the fossil close to the crown of *Lobaria*. According to the estimated ages, it is possible that the fossil represents *L. anomala*, which occurs from northern California to southern Alaska and appears to be an old species. Another possibility is that it represents *L. linita* (Ach.) Rabenh. or an ancestor of the cyanobacterial species in node *d* (*L. retigera*, *L. kurokawae* and *L. pseudopulmonaria*). Even though their mean ages were estimated at 26·9 Myr (node *a*) and 11·0 Myr (node *d*), respectively, the minimum age of node *a* and maximum age of node *d* are both estimated at *c.* 15 Myr (see Fig. 1). In any case, our time estimate rules out *L. pulmonaria* and *L. retigera* which also occur along the western Pacific coast but with much younger age estimates. The exclusion of *L. pulmonaria* is somewhat surprising, since the fossil has an elongated lobe that is common in green-algal species (node *e*) such as *L. pindarensis* or *L. yunnanensis* (Fig. 1, images on the right). However, the lack of soralia and the distribution range of most green-algal *Lobaria* species are arguments against extant green-algal species. Although the Bering land bridge connected Eurasia and North America resulting in one supercontinent and allowed the interchange of terrestrial biota throughout most of the Cenozoic Era, most green-algal species are currently found in restricted areas in East Asia (Yoshimura 1971). *Lobaria linita* has a circumpolar distribution but completely lacks soredia or isidia. Only *L. pulmonaria* has a worldwide distribution and produces soralia. All other green-algal species grow on tree bark in deciduous forests in restricted regions in South-East Asian mountains, temperate rainforests in northern East Asia, and the Himalayan subalpine forests. The first opening of the Bering Strait temporarily impeded land migrations towards the end of the Miocene 5·32 Myr ago (Marincovich & Gladenkov 1999; Gladenkov *et al.* 2002), indicating that most green-algal species evolved in East Asia after the initial separation of the supercontinent.

Considering all these arguments, we reject the hypothesis that the fossil impression belongs to *L. anomala* because the elongated lobe of the fossil differs from this species. Second, *L. pulmonaria* and *L. retigera* are ruled out due to their age, and *L. linita* due to the lack of vegetative propagules. Consequently, we hypothesize that the fossil impression belongs to an ancestral *Lobaria* species which formed elongated lobes and may have been associated with a green-alga or a cyanobacterium as the primary symbiont (node *b* or *e*). Although it is quite likely that the ancestral *Lobaria* produced soredia, we cannot reliably exclude the possibility that this fossilized individual produced clusters of isidia, whose marks on the stone are hardly distinguishable from those of soralia (node *d*).

Biogeographical context of the fossil

The hypothesis proposed here implies that the ancestral *Lobaria* may have been able to migrate through the Bering land bridge from North America to East Asia, or *vice versa*. According to Wen *et al.* (2016), beside the land bridge that joined both continents, floristic and faunal migration was primarily controlled by climate with 1) warm-temperate migration groups associated with the boreotropical-mixed mesophytic forests during the late Cretaceous to Paleogene (70–20 Myr BP), 2) boreal groups associated with taiga-coniferous forests in the Neogene (20–3 Myr BP), and 3) Arctic groups associated with tundra vegetation relatively recently during the Quaternary (3–0 Myr BP). There is considerable literature that demonstrates a close biogeographical connection between eastern Asia and western North America from the late Cretaceous to the late Neogene in major lineages of vascular plants (for a review, see Wen *et al.* 2014, 2016). Here, we wish only to highlight the genus *Picea* A. Dietr. which contains important substratum species for *Lobaria* taxa. Extensive studies of this genus show a disjunction similar to that in *Lobaria* with the highest species diversity found in the western Cordillera of North America and the Qinghai-Tibetan Plateau (QTP). The

diversification of the QTP-clade has been dated back to 19·8 Myr ago at the early Miocene. However, similar to the situation presented here, the ancestral area of the QTP-clade is still highly debated and may be interpreted as having a QTP or a North American origin (Ran *et al*. 2006; Bouillé *et al*. 2011; Lockwood *et al*. 2013).

Lobaria s. lat.

An issue that was not addressed in this study is whether species of closely related genera with similar characteristics might also fit the age of the fossil impression. The phylogeny presented in this paper follows the taxonomic revision by Moncada *et al*. (2013) that circumscribed *Lobaria* in a strict sense, separating the genera *Lobariella* Yoshim., *Lobarina* Nyl. ex Cromb., *Ricasolia* De Not. and *Yoshimuriella* B. Moncada & Lücking from *Lobaria*. Within these genera, there are many species that remain phylogenetically unverified and, thus, nomenclatorially unrevised. In what follows, we treat them as *Lobaria* s. lat. For example, *L. hallii* (Tuck.) Zahlbr. and *L. oregana* (Tuck.) Müll. Arg. are both present along the Pacific coast of North America and both have reticulated ridges. Of these two species, only *L. hallii* produces abundant soredia along ridges and lobe margins (and may possibly be related to the fossil impression) while *L. oregana* has lobulated vegetative propagules. Further work needs to be done to formalize the taxonomic positions of *L. hallii* and *L. oregana* within the genus *Lobarina* and to estimate the divergence age for this genus. Furthermore, a time analysis including all lineages of the family *Lobariaceae* could shed light onto the area of origin of the different genera and migration pathways.

Conclusions

Using a secondary calibration reference from *Parmeliaceae*, we have obtained the first dated phylogeny of *Lobaria* and estimated the ages of divergence of the mostly well-resolved species. The timetree gave a diversification estimate for *Lobaria* in the late Oligocene.

This timetree fits with the only known fossil from *Lobariaceae*, which is hypothesized here to be an ancestral *Lobaria* species. Analogous to East Asian-North American disjunct plants, the age estimates indicate that the paleoclimate and the closing or opening of the Bering Strait played a major role in shaping the current distribution of most *Lobaria* species. The ancestral *Lobaria* species must have successfully migrated along the Bering land bridge which acted as a major highway during warm-temperate climate periods but as a barrier during Arctic climate times. However, the time estimates presented here must be interpreted with caution owing to the lack of certainty regarding a linear relationship between mutation rates of *Parmeliaceae* and *Lobariaceae*. In addition, single long branches of some *Lobaria* species (*L. anomala*, *L. yunnanensis* and *Lobaria* sp. 1) suggest an under-sampling problem. While it can be assumed that some missing taxa are extinct, undescribed species from understudied regions are also conceivable.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit: https://doi.org/10.1017/S0024282917000676

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