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ARTICLE

Relationships of North American members of *Rhodiola* (Crassulaceae)

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Abstract: Taxa of *Rhodiola* L. (Crassulaceae) generally grow in arctic or alpine habitats. Some *Rhodiola* species are used medicinally, one taxon, *Rhodiola integrifolia* Raf. subsp. *leedyi* (Rosend. & J.W.Moore) Moran, (Leedy's roseroot), is rare and endangered, and the group's biogeography in North America is intriguing because of distributional disjunctions and the possibility that *Rhodiola rhodantha* (A.Gray) H.Jacobsen ($2n = 7_{II}$) and *Rhodiola rosea* L. ($2n = 11_{II}$) hybridized to form *Rhodiola integrifolia* Raf. ($2n = 18_{II}$). Recent studies of the North American *Rhodiola* suggest that the group's current taxonomy is misleading. We analyzed nuclear and chloroplast DNA sequences (internal transcribed spacer (ITS), *trnL* intron, *trnL–trnF* spacer, *trnS–trnG* spacer) from the North American *Rhodiola* taxa. We combined our data with GenBank sequences from Asian *Rhodiola* species, performed parsimony, maximum likelihood (ML), and Bayesian phylogenetic analyses, and applied a Bayesian clock model to the ITS data. Our analyses reveal two major *Rhodiola* clades, suggest that hybridization between *R. rhodantha* and *R. rosea* lineages was possible, show two distinct clades within *R. integrifolia*, and demonstrate that a Black Hills, South Dakota, *Rhodiola* population should be reclassified as Leedy's roseroot. We recommend that *R. integrifolia* be revised, and that the Black Hills Leedy's roseroot population be managed as part of that rare and endangered taxon.

Key words: biogeography, conservation, Crassulaceae, Leedy's roseroot, hybridization, *Rhodiola*.

Résumé : Le taxon des *Rhodiola* L. (Crassulaceae) croît normalement dans des habitats arctiques ou alpins. Certaines espèces de *Rhodiola* sont utilisées comme plantes médicinales et un taxon, *Rhodiola integrifolia* Raf. subsp. *leedyi* (Rosend. & J.W.Moore) Moran, (orpin de Leedy), est rare et menacé. La biogéographie de ce groupe en Amérique du Nord est intrigante à cause des disjonctions dans sa distribution et la possibilité que *Rhodiola rhodantha* (A.Gray) H.Jacobsen ($2n = 7_{II}$) et *Rhodiola rosea* L. ($2n = 11_{II}$) se soient hybridées pour former *Rhodiola integrifolia* Raf. ($2n = 18_{II}$). Des études récentes réalisées sur *Rhodiola* d'Amérique du Nord suggèrent que la taxonomie actuelle de ce groupe est erronée. Les auteurs ont analysé les séquences d'ADN nucléaire et chloroplastique (espaceur transcrit interne (ETI), intron *trnL*, espaceur *trnL–trnF*, et espaceur *trnS–trnG*) du taxon des *Rhodiola* d'Amérique du Nord. Ils ont combiné leur données avec les séquences des espèces de *Rhodiola* d'Asie tirées de GenBank, réalisé des analyses phylogénétiques de parcimonie, par maximum de vraisemblance et bayésienne, et appliqué un modèle d'horloge moléculaire bayésienne aux données de l'ETI. Leurs analyses révèlent l'existence de deux clades principaux de *Rhodiola*, suggèrent que l'hybridation entre les lignages *R. rhodantha* et *R. rosea* était possible, montrent l'existence de deux clades distincts à l'intérieur de *R. integrifolia* et démontrent qu'une population de *Rhodiola* de Black Hills, Dakota du Nord, devrait être re-classifiée en tant qu'orpin de Leedy. Ils recommandent que *R. integrifolia* soit révisé et que la population d'orpin de Leedy de Black Hills soit traitée comme faisant partie d'un taxon rare et menacé. [Traduit par la Rédaction]

Mots-clés : biogéographie, conservation, Crassulaceae, orpin de Leedy, hybridation, *Rhodiola*.

Introduction

Plant species in the genus *Rhodiola* L. are intriguing because of their biogeography, their uses in medicine, and their conservation requirements. The large majority of *Rhodiola* species are native to the alpine and arctic regions of Eurasia, and a few species are native to North America. *Rhodiola* species are perennial herbs with well-developed leafy or scaly rhizomes, usually with short (<25 cm) annual stems from the axils of their rhizome scales, but sometimes with annual stems that are up to 100 cm tall (Ohba 2003; Mayuzumi and Ohba 2004). Interest in pharmacological compounds from *Rhodiola* species is high, and traditional European and Chinese medicine has used extracts of *Rhodiola* plants for centuries to increase endurance, to decrease the effects of aging, and to treat a wide variety of illnesses (Cheng et al. 2012; Wang et al. 2012). In North America, the US Fish and Wildlife Service, and Minnesota and New York state agencies, are implementing plans to conserve *Rhodiola integrifolia* Raf. subsp. *leedyi* (Rosend. & J.W.Moore) Moran, which is very rare (US Fish and Wildlife Service 1998). Despite the importance of *Rhodiola* species, our understanding of their distributions, medicinal characteristics, and conservation needs has been hampered by our incomplete knowledge of the genus and its evolutionary relationships.

Carl Linnaeus first described the genus *Rhodiola* in 1753, recognizing it as distinct from *Sedum* (Moran 2000; Ohba 2003), but early 20th century authors such as Praeger regarded the genus as a synonym of *Sedum* L. (Ohba 1980), as did later authorities (e.g., Uhl 1952; Clausen 1975). The most recent evidence supports Linnaeus' classification of *Sedum* and *Rhodiola* as distinct genera (Ohba 1980, 2003; Mayuzumi and Ohba 2004), but there is still confusion about the species delimitations within *Rhodiola*, as illustrated by the fact that Ohba (2003) recognizes 58 species, while others recognize 90 or more species (Zhengyi and Raven 2001; Li and Zhang 2010). In this paper, we follow Ohba's (2003) taxonomy
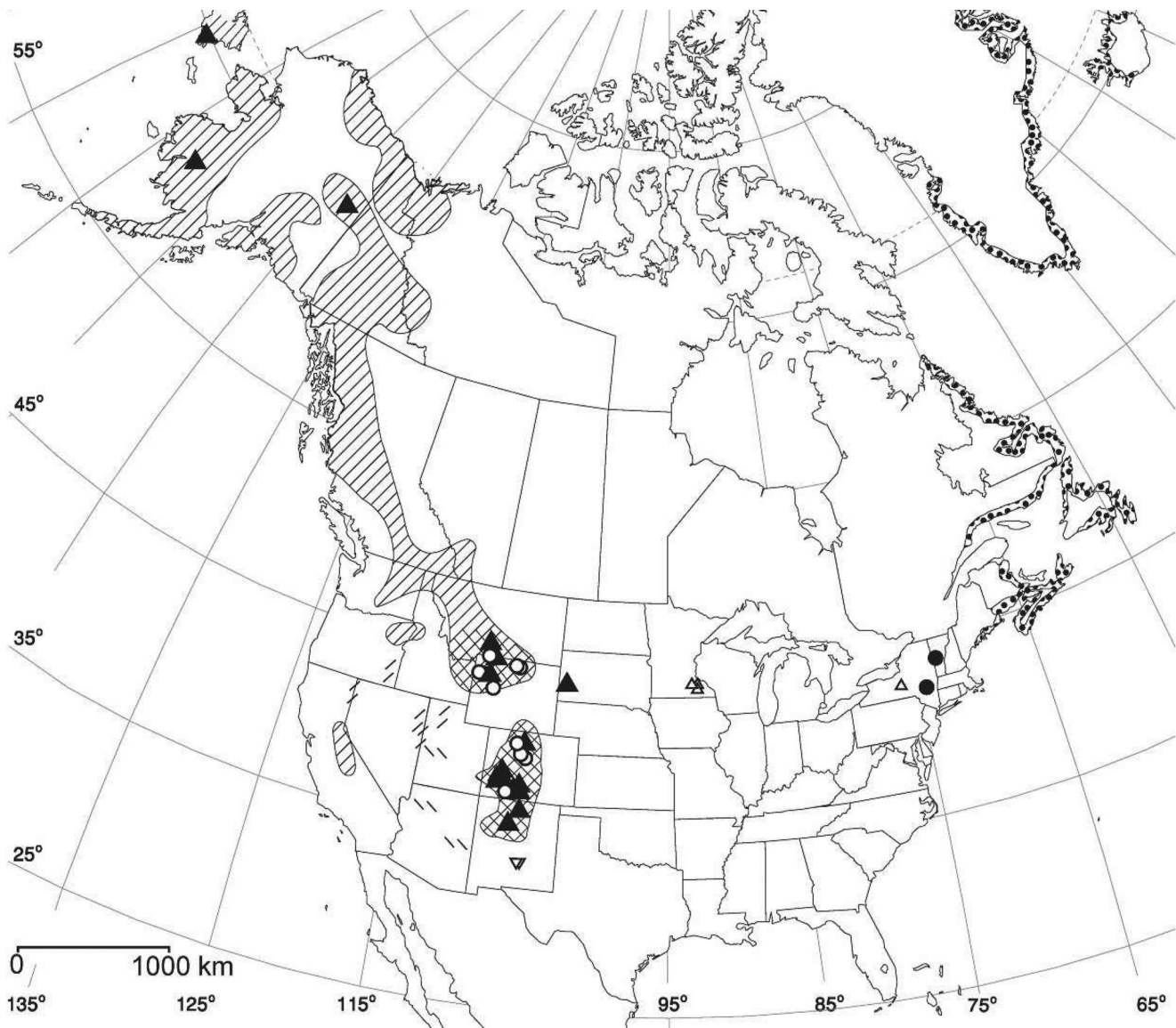
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Fig. 1. Approximate locations of sampled *Rhodiola* populations: *R. integrifolia* subsp. *integrifolia*, *R. integrifolia* subsp. *procera* or subspecies that were undetermined at time of collection (▲), *R. integrifolia* subsp. *leedyi* (△), *R. integrifolia* subsp. *neomexicana* (▽) *R. rhodantha* (O), *R. rosea* (●), and approximate known North American ranges of the widespread *Rhodiola* taxa: *R. integrifolia* subsp. *integrifolia* and *R. integrifolia* subsp. *procera* (//// hatching), *R. rhodantha* (\\\\ hatching), and *R. rosea* (dotted hatching). The *R. algida* population sample from the Katun River mountain range in Siberia is not pictured. Range data are from Clausen (1975) and the US Fish and Wildlife Service (1993).

of the genus because it is the most complete treatment, to our knowledge, and its treatment of the North American *Rhodiola* corresponds largely with that of the *Flora of North America* (Moran 2009) and with the data which we present in this paper.

According to Ohba (2003), *Rhodiola rhodantha* (A.Gray) H.Jacobsen, *Rhodiola integrifolia* Raf., and *Rhodiola rosea* L., are native to North America (Fig. 1). *Rhodiola integrifolia* and *R. rosea* are also native to Russia, and *R. rosea* is the only species with populations in North America, Asia, and Europe (Ohba 2003). *Rhodiola rhodantha* is restricted to the western US where it grows in moist meadows and on the edges of streams at elevations of 2000 to 4200 m a.s.l. (Fig. 1). It has a chromosome number of $2n = 7_{II}$, and produces an erect inflorescence of hermaphroditic pink flowers (Clausen 1975; Ohba 2003). *Rhodiola integrifolia* and *R. rosea* were considered closely related. Uhl (1952) and Clausen (1975) each discussed the possibility that *R. rosea* is a contributor to the *R. integrifolia* lineage, but they argued that any genetic contribution by *R. rosea* to *R. integrifolia* must have occurred outside North America, because

they believed that the species that might have hybridized were not present in North America at a time when they could have been in geographical contact. Zhang et al. (2014) suggest that *R. rosea* and *R. rhodantha* – *R. integrifolia* lineages colonized North America independently, and they call for dating and analyses of these colonization events.

Rhodiola integrifolia and *R. rosea* are morphologically similar, they are both described as dioecious, and they have even been treated as a single species by some authors. For example, Uhl (1952) called them "two chromosomal strains of *S. rosea*," and Gleason and Cronquist (1991) keyed them as two varieties of *S. rosea*. To clarify this taxonomy, Clausen (1975) conducted extensive field and common garden studies from the 1930s into the 1970s. Based on this work, Clausen argued that *R. integrifolia* and *R. rosea* are best understood as distinct species because their habitats and their chromosome numbers differ; *R. integrifolia* has $2n = 18_{II}$ and *R. rosea* has $2n = 11_{II}$ (Uhl 1952). Within *R. integrifolia*, Clausen (1975) interpreted his data as revealing two widespread, and two narrowly distributed

subspecies: (1) *Rhodiola integrifolia* Raf. subsp. *integrifolia*, (2) *Rhodiola integrifolia* subsp. *procera* (R.T.Clausen) H.Ohba, (3) *Rhodiola integrifolia* subsp. *neomexicana* (Britton) H.Ohba, and (4) *R. integrifolia* subsp. *leedyi*.

Most, but not all, recent literature follows Clausen's division of *R. integrifolia* into the four subspecies (e.g., US Fish and Wildlife Service 1998; Ohba 1999, 2003; Olfelt et al. 2001). The most widespread of the four subspecies is *R. integrifolia* subsp. *integrifolia*, ranging in North America from the southern Rocky Mountains, north to Alaska, and west to California (Fig. 1, Clausen 1975). Russian *R. integrifolia* populations are also classified as subspecies *integrifolia* according to Ohba (2003). The second most widespread subspecies is *R. integrifolia* subsp. *procera*, with populations throughout the southern Rocky Mountains, and one population in California. Because the range of *R. integrifolia* subsp. *procera* is entirely contained within the range of *R. integrifolia* subsp. *integrifolia*, and because Clausen did not consider some Alaskan *R. integrifolia* plants that closely match his description of *R. integrifolia* subsp. *procera*, Moran (2000) has questioned the validity of *R. integrifolia* subsp. *procera*. The two narrowly distributed *R. integrifolia* subspecies are *R. integrifolia* subsp. *neomexicana*, and *R. integrifolia* subsp. *leedyi*. *Rhodiola integrifolia* subsp. *neomexicana* populations are isolated on a single mountaintop in New Mexico (Clausen 1975) and are ranked by NatureServe (2013) as Critically Imperiled. Similarly, *R. integrifolia* subsp. *leedyi* (Leedy's roseroot) is a cliff-dwelling plant that is on the Minnesota and New York state endangered species lists and the US federal threatened species list (US Fish and Wildlife Service 1998). Because managing populations of rare and endangered species can be costly, US federal and state agencies have called for study of the distinctness of Leedy's roseroot from its relatives (US Fish and Wildlife Service 1993).

To test the genetic distinctness and variability of Leedy's roseroot, Olfelt et al. (2001) compared the North American *Rhodiola* species using 33 random amplified polymorphic DNA (RAPD) markers and 37 morphological markers in a large (1685 plants) common garden experiment. The study included *Rhodiola* populations from Colorado, New York, New Mexico, Minnesota, and Vermont, but no *R. integrifolia* representatives from the central and northern Rockies, Alaska, or Russia. The phenetic analyses of the RAPD and common garden data clustered *R. rhodantha* and *R. integrifolia* closely to one another and *R. rosea* relatively distantly from them. The RAPD and common garden data also demonstrated that Leedy's roseroot is genetically distinct from its relatives in the southern Rockies, and suggested that the taxon's differences from *R. integrifolia* subsp. *integrifolia*, *R. integrifolia* subsp. *procera*, and *R. integrifolia* subsp. *neomexicana* might best be interpreted as differences between species.

We tested the hypothesis that the phenetic analyses of North American species of *Rhodiola* would reflect their phylogenetic relationships and we used a molecular clock model to test the hypothesis that *R. rhodantha* and *R. rosea* were both present in North America early enough for their ranges to come into contact to allow a hybridization event and produce the *R. integrifolia* lineage, as suggested by Hermsmeier et al. (2012). We used nuclear ribosomal internal transcribed spacer (ITS) and chloroplast (*trnL* intron, *trnL*–*trnF* spacer, *trnS*–*trnG* spacer) DNA sequence data because of their demonstrated utility in revealing relationships among recently diverged taxa and because they are thought to evolve independently (Baldwin et al. 1995; Mort et al. 2007; Jia et al. 2012). We sequenced DNA from representatives of the Colorado, New Mexico, New York, Minnesota, and Vermont populations sampled in the Olfelt et al. 2001 study, and we collected new representatives from South Dakota, Montana, Wyoming, Alaska, and Russia. Finally, we tested the phylogenetic position of the North American *Rhodiola* in relationship to many of the Asian *Rhodiola* species using GenBank sequence data (Appendix A).

Methods

Taxon sampling

We sampled a total of 108 individuals representing 31 different populations of the six named North American *Rhodiola* taxa. Specimens were chosen to encompass the North American range of each taxon, except for *R. rosea*, which was represented by individuals from two populations: one in New York, and the other in Vermont near the southern limit of the species' range (Fig. 1). We focused our collections on the western North American taxa because preliminary analyses, which included the sequence data from Mayuzumi and Ohba (2004), suggested that *R. rhodantha* and *R. integrifolia* formed a monophyletic clade and because analysis of such a group seemed likely to be useful for conservation decisions. All North American material was collected by the authors in 1994, 1995, or 2012, except for two Alaskan specimens of *R. integrifolia* that were obtained from the University of Alaska Museum of the North Herbarium (ALA). To place the North American *Rhodiola* taxa into their genus-wide context, we obtained two Asian *R. integrifolia* and one *Rhodiola algida* (Ledeb.) Fisch. & C.A.Mey. specimens from ALA for DNA extraction and sequencing, and we obtained sequences of Eurasian *Rhodiola* and *Pseudosedum* A.Berger taxa from GenBank (Appendix A). The *Pseudosedum* species were selected as outgroups based on the subfamily-level analysis of the Eastern Asian Sedoideae (Crassulaceae) by Mayuzumi and Ohba (2004). For a complete list of accessions, including voucher data, population locations, and GenBank accession numbers, see Appendix B.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from field-collected specimens following the cetyltrimethylammonium bromide (CTAB) protocol (Davis et al. 1995) as modified by Olfelt et al. (1998), or using DNeasy Plant Mini Kits (QIAGEN, Valencia, California, USA) following the manufacturer's instructions. Genomic DNA from herbarium specimens was extracted using a CTAB method developed for dried leaves (Cota-Sánchez et al. 2006). Genomic regions were amplified using polymerase chain reaction (PCR) with the ITS primers 26S25R and ITS5* of Liston et al. (1996), *trnL*–*trnF* and *trnL* primers E, F, C, and D of Taberlet et al. (1991), and *trnS*–*trnG* primers trnS^{GCU} and 5′trnG2S of Shaw et al. (2005). PCR reactions were done in 50 μL volumes containing 5 μL of 5× Taq Master (5 PRIME Inc., Hamburg, Germany), 5 μL of 10× PCR buffer (5 PRIME Inc.), 4 μL of 2.5 mmol·L^{-1} dNTP mix (Eppendorf Scientific Inc., Hamburg, Germany), 1 μL each of 20 pmol·μL^{-1} forward and reverse primers (Fisher Scientific Corp., Pittsburg, Pennsylvania, USA), 0.2 μL of 5 U·μL^{-1} Taq DNA polymerase (5 PRIME Inc.), 32.8 μL of Milli-Q H$_2$O (Millipore Corp., Billerica, Massachusetts, USA), and 1 μL of ~1–10 ng·μL^{-1} genomic DNA. Amplification of the ITS region was carried out with the PCR parameters 94 °C, 1 min; 35 × (94 °C, 1 min; 55 °C, 1 min; 72 °C, 3 min); 72 °C, 7 min. For amplification of the *trnS*–*trnG* spacer we used the following parameters: 80 °C, 1 min; 35 × (95 °C for 1 min; 50 °C for 1 min with a ramp of 0.3 °C·s^{-1}; 65 °C for 5 min); 65 °C for 10 min. For amplification of the *trnL* intron and *trnL*–*trnF* spacer we used the following thermal cycle parameters: 94 °C for 3 min; 35 × (94 °C for 1 min; 50 °C for 1 min; 72 °C for 2 min); 72 °C for 5 min. Amplifications were performed on an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf Scientific Inc.). We verified the presence and approximate size of PCR products using 1.4% agarose gels stained with SYBR Green I (Invitrogen, Carlsbad, California, USA). The PCR products were then purified using Wizard PCR Preps DNA Purification Kits (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's instructions. Forward and reverse DNA sequencing was performed with ABI Prism DNA sequencers at either the University of Minnesota's Advanced Genetic Analysis Center or SeqWright, Inc. (Houston, Texas, USA).

Fig. 2. The Bayesian inference majority rule consensus phylogram of the *Rhodiola* combined data matrix with Bayesian support, maximum likelihood, and parsimony bootstrap values on each branch. Dashes represent nodes that were not present owing to weakly supported (<0.5 Bayesian posterior probability, maximum likelihood bootstrap, or parsimony bootstrap value) incongruences with the Bayesian cladogram.

Phylogenetic analysis

Sequences were aligned with the program ClustalW version 2 (Larkin et al. 2007) and phylogenetic analyses were performed on three data matrices (ITS, chloroplast, and combined) using maximum parsimony, maximum likelihood (ML), and Bayesian inference methods. The combined data matrix was constructed by concatenating the aligned data for all samples that had sequences for both the ITS and at least one of the chloroplast loci (5–21 individuals for each of the six North American *Rhodiola* taxa). The aligned data matrices are available on TreeBASE (study number 14854).

ML analyses were performed using RAxML version 7.4.4 (Stamatakis 2006; Stamatakis et al. 2008) with 100 rapid bootstrap iterations followed by a thorough ML search. The ML analysis utilized the GTR+Γ nucleotide substitution model, and the analysis of the combined data matrix used separate partitions for the ITS and chloroplast data. For the Bayesian analysis, the optimal models of molecular evolution for the ITS and chloroplast loci were selected using the Akaike Information Criteria (AIC, Akaike 1974), as implemented in jModelTest (Posada 2008). The Bayesian analyses were conducted in MrBayes 3.2 (Ronquist et al. 2012) with the two *Pseudosedum* taxa set as a rooting constraint. The analysis of the combined data matrix was partitioned to make use of the substitution models identified by jModelTest. Two independent runs of four chains each (one cold, three heated) were performed for 5 000 000 generations, with parameters sampled every 500 generations. The runs were configured with the default flat priors, and the first 25% of trees were discarded as burn-in. Convergence was determined by confirming that the average standard deviation of split frequencies was less than 0.01 and that all potential scale reduction factor (PSRF) values = 1.00. Maximum parsimony analyses were done in MEGA 5.05 (Tamura et al. 2011) using the heuristic Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) on a set of 10 random initial trees with 1000 bootstrap replicates (Felsenstein 1985).

Divergence time estimates

To compare the likelihood of a relaxed clock model with the likelihood of a strict clock model with the ITS data set, we used the stepping-stone sampling method (Xie et al. 2011) as implemented in MrBayes. We then estimated divergence times using the ITS data set with the preferred clock model in BEAST version 2.0.1 (Drummond et al. 2012) with the mean ITS substitution rate (μ) found for *Aichryson* (Crassulaceae) of 5.69×10^{-3} substitutions per site per million years (Mes et al. 1996), which was successfully used to estimate divergence times for other *Rhodiola* taxa (Gao et al. 2012) and *Pedicularis* (Yang et al. 2008). The divergence time analysis was repeated with $\mu = 3.46 \times 10^{-3}$ substitutions per site per million years and $\mu = 8.69 \times 10^{-3}$ substitutions per site per million years to cover the range of ITS substitution rates reported for shrubs and herbaceous plants in Richardson et al. (2001). We used the substitution model selected by jModelTest and a Yule process speciation model as a Bayesian prior probability. Trees were sampled every 1000 generations for 10 000 000 generations and the first 2500 trees were discarded as burn-in. BEAST parameter output was assessed for convergence in Tracer version 1.6 (Rambaut et al. 2013) by ensuring that the effective sample size for all parameters was above 200. The program TreeAnnotator version 1.6.1 (Rambaut and Drummond 2007) was used to summarize the posterior estimates and highest posterior density (HPD) limits of the node heights from the sample trees and produce a maximum clade credibility chronogram. We then used FigTree version 1.4.0 (Rambaut and Drummond 2009) to visualize the consensus chro-

nogram showing mean divergence time estimates with 95% HPD intervals.

Results

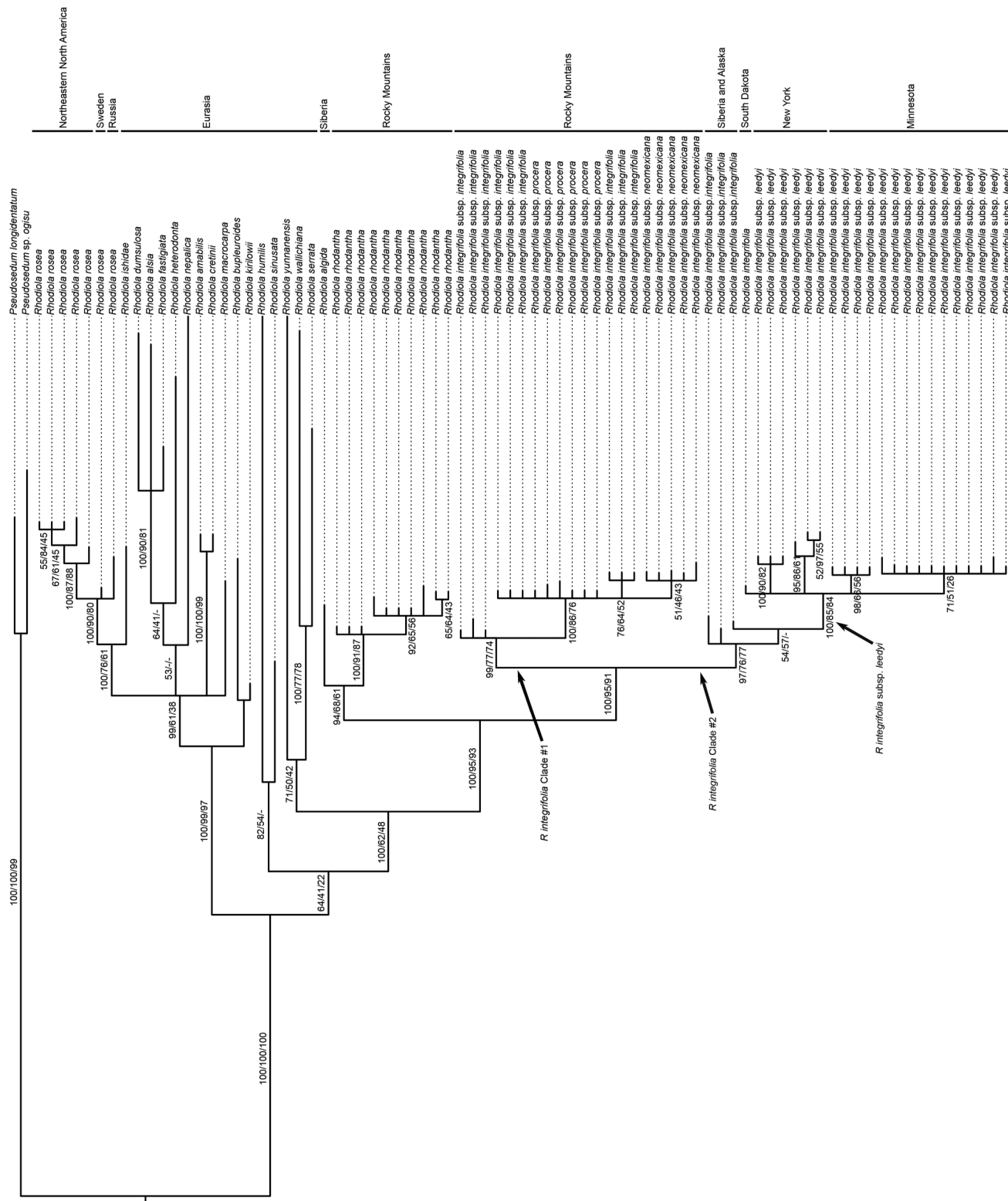### Sequence data and alignments

A total of 284 new sequences were produced for this study and are available in GenBank (Appendix B). The aligned ITS data matrix comprised 681 characters, of which 158 (23%) were variable and 82 (12%) were parsimony informative. The optimal model of molecular evolution for the ITS data set was identified as TIM1ef+Γ by jModelTest. The chloroplast data matrix consisted of the concatenated *trnL*, *trnL–trnF*, and *trnS–trnG* sequences. The alignment contained 1649 nucleotides, with 61 (4%) variable sites and 42 (3%) parsimony informative sites. The DNA evolution model selected for the chloroplast data matrix by jModelTest was the TIM1+Γ substitution model. The combined ITS and chloroplast data set was 2330 characters long, with 193 (8%) variable sites of which 115 (5%) were parsimony informative.

Phylogenetic analyses

All analyses of the ITS data set yielded well-resolved phylogenies, however the chloroplast trees were poorly resolved and had no strongly supported (>0.5 Bayesian posterior probability, ML bootstrap, and parsimony bootstrap values) incongruences with the ITS tree. The combined data set produced phylogenies that did not differ in topology from the ITS phylogeny; the only differences were increases in resolution and clade support. The consensus topologies from the parsimony, ML, and Bayesian analyses were identical except for weakly supported nodes (<0.5 Bayesian posterior probability, ML bootstrap, and parsimony bootstrap values), thus we show here only the Bayesian majority rule consensus phylogram of the combined data matrix with Bayesian posterior probability, ML bootstrap, and parsimony bootstrap values on each branch (Fig. 2).

Our analyses reveal two major *Rhodiola* clades with variable support. *Rhodiola rosea* from northeastern North America, Sweden, and Russia nest within a well-supported clade that also contains the 11 Eurasian *Rhodiola* species, *R. ishidae*, *R. dumulosa*, *R. alsia*, *R. fastigiata*, *R. heterodonta*, *R. nepalica*, *R. amabilis*, *R. cretinii*, *R. macrocarpa*, *R. bupleroides*, and *R. kirilowii* (100/99/97; Bayesian posterior probability, ML bootstrap, and maximum parsimony bootstrap values) (Fig. 2). The North American and Eurasian representatives of *R. rosea* form a clade sister to *R. ishidae*, and appear to be only distantly related to *R. rhodantha* and *R. integrifolia*. These two taxa, the first one, which is strictly North American, and the second one, which is both North American and Asian, are nested in a clade (64/41/22) that also contains the six Eurasian (or Siberian) species *R. humilis*, *R. sinusata*, *R. yunnanensis*, *R. wallichiana*, *R. serrata*, and *R. algida*. The Siberian *R. algida* is sister to the Rocky Mountain endemic *R. rhodantha*, and together these form a clade (94/68/61) sister to *R. integrifolia*.

Rhodiola integrifolia is strongly supported as monophyletic (100/95/91) with two moderate to well-supported clades: one largely northern (97/76/77), and the other largely southern (99/77/74). The largely southern clade is labeled "*R. integrifolia* Clade #1" in Fig. 2, and contains all populations of *R. integrifolia* subsp. *integrifolia* from the central and southern Rocky Mountains, as well as all populations of *R. integrifolia* subsp. *procera* and *R. integrifolia* subsp. *neomexicana*. *Rhodiola integrifolia* subsp. *procera* fails to resolve separately from *R. integrifolia* subsp. *integrifolia*, and *R. integrifolia* subsp. *neomexicana* forms a weakly supported clade (51/46/43). The largely northern clade within *R. integrifolia* contains all populations of *R. integrifolia* subsp. *integrifolia* from Siberia and Alaska, as

well as the New York and Minnesota populations of *R. integrifolia* subsp. *leedyi* (*R. integrifolia* Clade #2, Fig. 2). The small geographically isolated population on Harney Peak in the Black Hills of South Dakota nests with *R. integrifolia* subsp. *leedyi* in a strongly supported clade (100/85/84).

Divergence time estimates

The autocorrelated relaxed clock model with log-normally distributed rates (Thorne and Kishino 2002) was preferred over the strict clock model by over 20 natural log likelihood units; a greater than 5 unit difference is considered very strong evidence in favor of the better model (Kass and Raftery 1995). The relaxed clock model allowed substitution rates among lineages to vary in an autocorrelated manner, so that the rate of each branch came from a lognormal distribution whose mean was a function of the parent branch's rate. The Bayesian inference consensus chronogram produced from the ITS data set using the substitution rate from *Aichryson* (Crassulaceae) can be seen in Fig. 3. The nodes represent posterior mean ages and the gray node bars are the 95% HPD intervals.

Our analyses reveal that two separate lineages of *Rhodiola* colonized North America an estimated 2.0 and 0.7 million years ago (mya). Using the substitution rate from *Aichryson* (Crassulaceae), the lineage that led to western North American *R. rhodantha* and *R. integrifolia* diverged from the Eurasian *R. algida* approximately 2.0 mya ($\mu = 5.69 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.87–3.15 mya, 95% HPD estimates). ITS substitution rates from more distantly related plants placed the estimate between 1.3 mya ($\mu = 8.69 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.62–2.06 mya, 95% HPD estimates) and 3.2 mya ($\mu = 3.46 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 1.46–5.19 mya, 95% HPD estimates). The northeastern North American *R. rosea* diverged from the Swedish and Russian *R. rosea* approximately 0.7 mya (0.14–1.26 mya, 95% HPD estimates), or between 0.44 mya ($\mu = 8.69 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.11–0.85 mya, 95% HPD estimates) and 1.07 mya ($\mu = 3.46 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.29–2.06 mya, 95% HPD estimates). Furthermore, two clades within *R. integrifolia* diverged from each other an estimated 1.0 mya ($\mu = 5.69 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.33–1.67 mya, 95% HPD estimates), or between 0.63 mya ($\mu = 8.69 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.11–0.74 mya, 95% HPD estimates) and 1.61 mya ($\mu = 3.46 \times 10^{-3}$ substitutions·site^{-1}·(million years)$^{-1}$, 0.59–2.83 mya, 95% HPD estimates). The clade represented as Rocky Mountains *R. integrifolia* subsp. *integrifolia* in our chronogram (Fig. 3) contains all of the southern and central Rocky Mountain populations of *R. integrifolia* subsp. *integrifolia*, *R. integrifolia* subsp. *procera*, and *R. integrifolia* subsp. *neomexicana* (which all have homogenous ITS sequences), and the other clade contains *R. integrifolia* subsp. *leedyi* as well as all Siberian and Alaskan populations of *R. integrifolia* subsp. *integrifolia*.

Discussion

Our analyses show that *R. rosea* (Northeastern North America, Sweden, and Russia) nests within a well-supported clade (100/99/97; Bayesian posterior probability, ML bootstrap, and maximum parsimony bootstrap values, Fig. 2) along with 11 strictly Eurasian species. The North American species *R. rhodantha*, and the North American and Siberian *Rhodiola integrifolia* form a separate clade (64/41/22) with six other species labeled Siberia, Rocky Mountains, Siberia and Alaska, South Dakota, New York, and Minnesota in Fig. 2. Within this clade, *R. integrifolia* forms a sister clade (100/95/91) to *R. rhodantha* and *R. algida* (94/68/61), thus the analyses support our working hypothesis that *R. rhodantha* and *R. integrifolia* are closely related and that *R. rosea* is relatively distantly related to them. Our data also show that there are two lineages within *R. integrifolia*, one largely southern (99/77/74; labeled *R. integrifolia* Clade #1 in Fig. 2) and one largely northern (97/76/77; labeled *R. integrifolia* Clade #2), and that Leedy's roseroot belongs to the largely north-
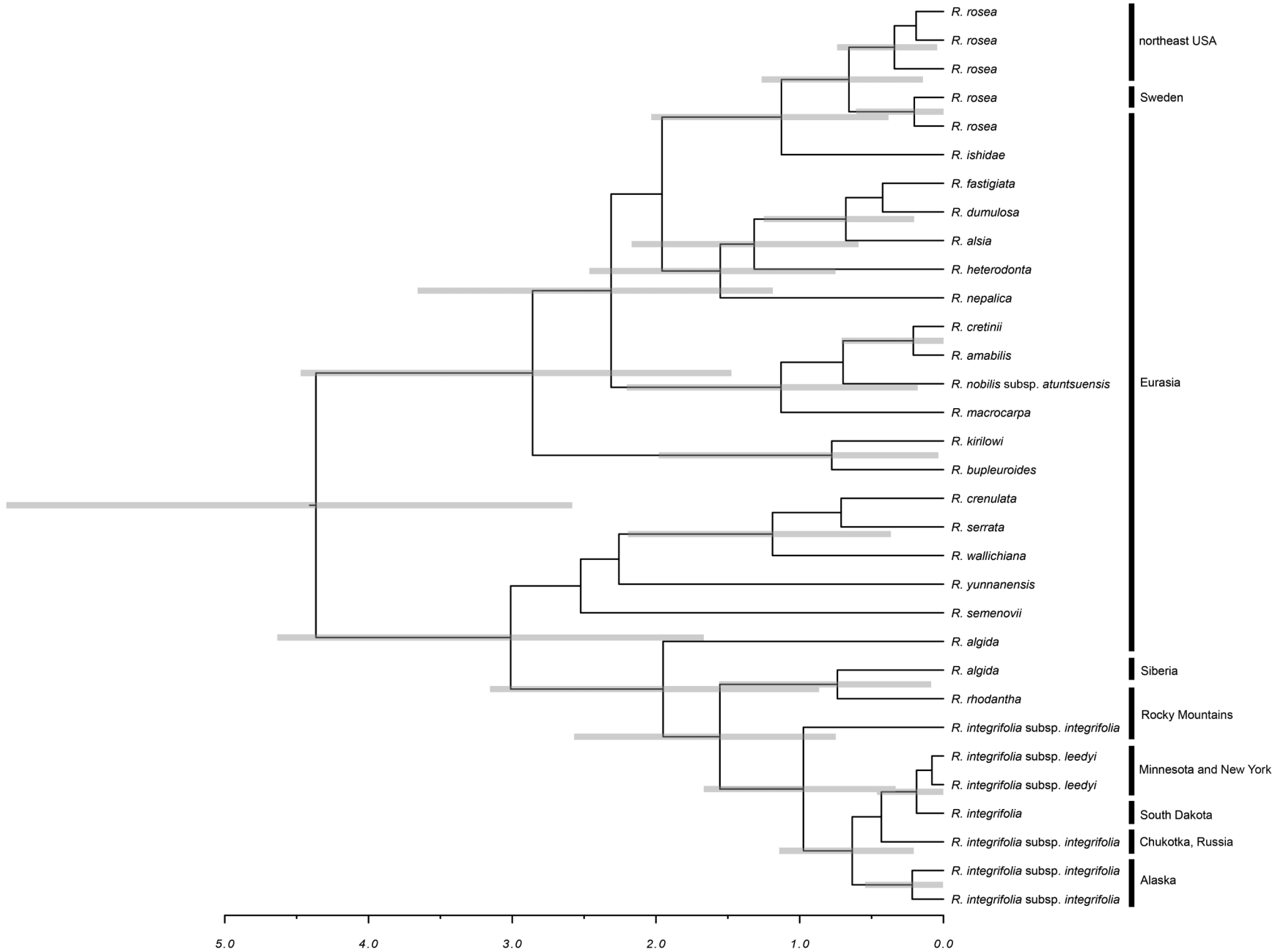
ern clade. Lastly, our data show that the *Rhodiola* lineage that gave rise to *R. algida* and *R. rhodantha* diverged approximately 2 mya, and that the European and North American *R. rosea* populations in our analyses diverged an estimated 0.7 mya (Fig. 3).

These estimated divergence dates suggest that the *R. rosea* and *R. rhodantha* lineages have been present in North America since at least the Middle Pleistocene. During much of the Pleistocene, between 2 mya and 0.7 mya, glaciers covered eastern and western North America, but not a broad central corridor from the Arctic Circle southwards, and not Beringia. Later in the Pleistocene, glaciers covered the central corridor as well (Barendregt and Duk-Rodkin 2011). These glaciations would have formed potentially suitable habitats for multiple *Rhodiola* species near their margins, and would have formed corridors for potential east–west migration of arctic and alpine species. Given these potential habitats and the complex range shifts in arctic and alpine species that have been documented for the Pleistocene (Brochmann and Brysting 2008; Allen et al. 2012), overlap in *R. rhodantha* ($2n = 7_{II}$) and *R. rosea* ($2n = 11_{II}$) ranges could have occurred to produce *R. integrifolia* ($2n = 18_{II}$). Our estimated divergence dates for the North American *Rhodiola* species, and the glaciation patterns of the Pleistocene thus support the assertion of Hermsmeier et al. (2012), based on their nuclear genetic data, that *R. integrifolia* is the result of a *R. rhodantha* – *R. rosea* hybridization event. Further evidence for such a hybridization event comes from Guest's (2010) finding of matching *R. integrifolia* and *R. rhodantha* chloroplast haplotypes for several Colorado and Wyoming populations of the two species.

The separation of the Rocky Mountain *R. integrifolia* Clade #1 populations from those in the northern clade (100/95/91) is in agreement with analyses by Guest (2010). Guest studied ITS and chloroplast sequences in *R. integrifolia* populations from Alaska, British Columbia, and the western continental US states excluding South Dakota, and found sequence divergence among the populations depending on whether they are north or south of approximately 49° latitude. The division of *R. integrifolia* into the largely northern and largely southern clades (97/76/77 and 99/77/74) and their estimated time of divergence of 1 mya suggest that *R. integrifolia* might best be described as two species. In fact, Britton and Rose (1903) described such a species, *R. alaskana*, as "resembling *R. integrifolia*, but usually taller and more slender," based on a type specimen from Misty Harbor on Nagai Island, Alaska. Clausen (1975) treated *R. alaskana* as a synonym of *R. integrifolia* (which he included in the genus *Sedum*), and described plants from Alaska, the Yukon, and Mackenzie as a distinctive but morphologically indistinguishable ecotype of *R. integrifolia* subsp. *integrifolia*. He further noted that plants from Alaska did not live long enough in his common garden plots to yield morphological data for comparison with plants from other locations, and he suggested that this failure to survive might be because of underlying differences in physiology from other *R. integrifolia* populations. Our data may support Britton and Rose's differentiation of *R. alaskana* and correspond with Clausen's distinctive *R. integrifolia* subsp. *integrifolia* ecotype from Alaska. In any case, our data show that the taxonomy within *R. integrifolia* as treated by Ohba (2003) needs revision.

Within the Rocky Mountain *R. integrifolia* clade, there is no support for separate clades corresponding to *R. integrifolia* subsp. *integrifolia* and *R. integrifolia* subsp. *procera*. This is in agreement with the low levels of genetic differentiation between *R. integrifolia* subsp. *integrifolia* and *R. integrifolia* subsp. *procera* revealed in the RAPD and common garden data study of the taxa by Olfelt et al. (2001). In addition, the ranges of *R. integrifolia* subsp. *integrifolia* and *R. integrifolia* subsp. *procera* overlap extensively, so our analyses agree with Moran's (2009) treatment of *R. integrifolia* in the *Flora of North America* in which *R. integrifolia* subsp. *procera* is reduced to synonymy under *R. integrifolia* subsp. *integrifolia*. While there is only weak phylogenetic support (51/46/43) for a clade corresponding to *R. integrifolia* subsp. *neomexicana*, both morphological and

Fig. 3. Chronogram of *Rhodiola* with Bayesian inference divergence time estimates. Nodes are posterior mean ages (million years ago) and gray node bars represent the 95% highest posterior density (HPD) intervals.

geographical data support *R. integrifolia* subsp. *neomexicana* as distinct (Olfelt et al. 2001), so we recommend that the taxon be maintained. *Rhodiola integrifolia* subsp. *neomexicana* is a narrow endemic restricted to high elevations (3500 m a.s.l.) atop Sierra Blanca Peak of Otero County, New Mexico (Clausen 1975). This taxon may be endangered by road construction, recreational activities, and altitudinal shifts in vegetation because of climate change (Halloy and Mark 2003; Walther et al. 2005). We know of no active conservation efforts for *R. integrifolia* subsp. *neomexicana*, which warrants active monitoring and conservation by the concerned New Mexico and southwestern US agencies, and collection of seeds for deposit in a seed bank.

The close relationship of the Leedy's roseroot and Harney Peak populations with the Alaskan and Siberian *R. integrifolia*, and the estimated time of divergence of the *R. integrifolia* clades of 1 mya (Fig. 3) suggest that populations of an ancestral *R. integrifolia* lineage became separated into two refugia during one of the Early or Middle Pleistocene glacial maxima. One of the refugia might have been in the central or southern Rocky Mountains and the other in Beringia (Duk-Rodkin et al. 2004; DeChaine and Martin 2005). The patterns of sequence variation evident in Guest's analysis and in our data set suggest that Leedy's roseroot and the Harney Peak (South Dakota), Canadian, Alaskan, and Siberian *R. integrifolia* populations originate from the Beringian refugium.

The Harney Peak (South Dakota) *R. integrifolia* population, which was not identified to subspecies in the herbarium sheets that we consulted (RM accessions 698922, 698947, 698964), clearly shares a most recent ancestor with all of the Leedy's roseroot populations. Like the Minnesota Leedy's roseroot populations, the Harney Peak plants grow on a moist north-facing cliff, have a trailing rather than an upright growth habit, and cluster in rock fissures that are moist with seep water. In the case of the Harney Peak population, this water may come from a pool located approximately 30 m above and 70 m south of the population (J. Olfelt, personal observation, 2012). This population should be classified and managed as Leedy's roseroot along with the Minnesota and New York populations.

The Minnesota and New York populations of Leedy's roseroot are recognized under Minnesota, New York, and US federal law as threatened or endangered. The data presented here demonstrate that there are important molecular evolutionary divergences between Leedy's roseroot (including the South Dakota population) and its central and southern Rocky Mountain relatives. The closer than expected relationships revealed between Leedy's roseroot and the Alaskan and Siberian *R. integrifolia* show that Leedy's roseroot may not be as divergent from its northwestern relatives as we hypothesized based on the Olfelt et al. (2001) phenetic data. However, we highlight Clausen's (1975) observation that the environmental conditions of Alaska and Siberia differ so dramatically from those surrounding the Leedy's roseroot populations that the Alaskan *R. integrifolia* individuals could not survive near the latitudes at which Leedy's roseroot populations grow. There are apparently critical differences between the northwestern *R. integrifolia* and Leedy's roseroot that demonstrate the need for continued protection for Leedy's roseroot populations under the US Endangered Species Act.

The biodiversity of a group, which is usually defined by the number of species in the group, is best understood when it is explored via multiple approaches (Baldwin 2000; Sites and Marshall 2004; Devey et al. 2008). Here we have presented phylogenetic evidence that *R. rosea*, *R. rhodantha*, and *R. integrifolia* each belong to separate monophyletic groups, and that within *R. integrifolia* there are at least two separately evolving lineages. This study should be followed up with a morphological study that includes samples of populations from across the entire range of *R. integrifolia*, as described by Ohba (2003). Such a study should be designed to ensure that sufficient representatives are included from Alaska, Canada, and Siberia to rigorously test whether morpho-

logical variation corresponds with the DNA sequence data presented in this paper. The morphological data along with the available molecular data have the potential to yield robust evidence of the taxonomic groups that will be evolutionarily meaningful and useful both for field identifications and for the development of conservation plans.

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Appendix A

List of Eurasian *Pseudosedum* and *Rhodiola* sequences obtained from GenBank. The information is listed as follows: **taxon**; then GenBank accession numbers for the sequences (*ITS*, trnL intron, **trnL-trnF**, *trnS-trnG*).

Pseudosedum longidetatum; AB088609.1, AB089762.1 **P. sp. ogisu**; *AB088610.1,* **AB089763.1. Rhodiola algida**; *JQ228604.1.* **R. alsia**; HQ841011.1, HQ840991.1. **R. amabilis**; *AB088587.1,* **AB089760.1. R. bupleuroides**; *JF978172.1,* **AB089750.1. R. chrysanthemifolia**; *AB088606.1,* **AB089756.1. R. cretinii**; *AB088588.1,* **AB089761.1. R. crenulata**; *JF978174.1.* **R. dumulosa**; *AB088595.1,* FJ794313.1, **FJ794313.1. R. fastigiata**; *JF978176.1,* **AB089749.1. R. heterodonta**; *AB088596.1,* **AB089745.1. R. humilis**; *AB088611.1,* **AB089755.1. R. ishidae**; *AB088600.1,* **AB089751.1. R. kiriowii**; *JF978180.1,* FJ974038.1, **FJ974038.1. R. macrocarpa**; *AB088590.1,* **AB089759.1. R. nobilis sp. atuntsuensis**; *AB088589.1,* **AB089758.1. R. nepalica**; *AB088598.1,* **AB089754.1. R. rosea**; *GQ374201.1, GQ374199.1,* GQ374226.1, GQ374228.1, **GQ374226.1, GQ374228.1. R. semenovii**; *JQ228603.1.* **R. serrata**; *AB088597.1,* **AB089747.1. R. sinuata**;

AB088605.1, **AB089753.1**. **R. wallichiana**; *AB088607.1*, **AB089757.1**. **R. yunnanensis**; *EU239673.1*, **AB089748.1**.

Appendix B

List of *Rhodiola* taxa and populations investigated. The information is listed as follows: **taxon**; then for each population of the taxon sampled, *locality of population*, collection number (herbarium acronym) and GenBank accessions for the population (*ITS*, trnL intron, **trnL-trnF**, *trnS-trnG*).

Rhodiola algida (Ledeb.) Fisch. & C.A.Mey: *Katun River Mountain Range Altai Republic,* V80794 (ALA) *KF875763, KF875969,* **KF875838**; **Rhodiola integrifolia subsp.** undetermined at time of sampling, *Harney Peak SD USA,* 17989 (CHIC) *KF875780, KF875966,* **KF875844**, *KF875907*; **Rhodiola integrifolia Raf. subsp. integrifolia**: *Sandia Crest NM USA,* 519442 (MIN), *FJ769168, KF875730, KF875930,* **KF875804**, *KF875863; Rio Media NM USA,* 519396 (MIN), *KF875731, KF875931,* **KF875805**, *KF875864, KF875732, KF875932,* **KF875806**, *KF875865; Wolf Creek Pass CO USA,* 432098 (MIN), *KF875733, KF875734, KF875935,* **KF875807**, *KF875866, KF875735; Milner Pass CO USA,* 519568 (MIN) *KF875736, KF875933,* **KF875808**, *KF875867, KF875737, KF875738, KF875934,* **KF875809**, *KF875868; Beartooth Highway MT USA,* 17998 (CHIC) *KF875779, KF875965,* **KF875842**, *KF875906; Benbow Road MT USA,* 17999 (CHIC) *KF875778, KF875964,* **KF875840**, *KF875905; Torrey Creek WY USA,* 17993 (CHIC) *KF875781, KF875967,* **KF875841**, *KF875908; Chukotka Peninsula Russia,* V113690 (ALA) *KF875782, KF875963,* **KF875851**, *KF875904; Southwestern AK USA,* V163027 (ALA) *KF875783,* **KF875839**; *Eastern AK USA,* V151939 (ALA) *KF875784, KF875968,* **KF875850**, *KF875903;* **Rhodiola integrifolia subsp. neomexicana (Britton) H.Ohba,** *Ice Springs-Sierra Blanca, NM USA,* 519395 (MIN), *FJ769170,* **KF875936**, *KF875748, KF875937, KF875870, KF875749, KF875938,* **KF875811**, *KF875871, KF875750, KF875751,* **KF875812**, *KF875872,* **KF875810**, *KF875869; Apache Ski Area-Sierra Blanca, NM USA,* 519457 (MIN) *KF875752, KF875939,* **KF875813**, *KF875874, KF875753, KF875754, KF875940,* **KF875814**, *KF875875;* **Rhodiola integrifolia subsp. procera (R.T.Clausen) H.Ohba**: *Elwood Pass, CO USA,* 430366 (MIN), *FJ769169, KF875740, KF875941,* **KF875815**, *KF875876; Eureka Gulch, CO USA,* 519445 (MIN) *KF875746, KF875943,* **KF875817**, *KF875877, KF875742; Picayune Gulch CO USA,* 519569 (MIN) *KF875743, KF875944, KF875744,* **KF875818**; *Molas Pass, CO USA,* 519444 (MIN) *KF875745, KF875945,* **KF875819**, *KF875878; Los*

Animas River, CO USA, 519443 (MIN) *KF875747, KF875946,* **KF875820**, *KF875879;* **Rhodiola integrifolia Raf. subsp. leedyi (Rosend. & J.W.Moore) Moran:** *Whitewater Wildlife Management Area, MN USA,* 456701 (MIN), *FJ769162, KF875913,* **KF875792**, *KF875894, KF875717, KF875718, KF875914,* **KF875793**, *KF875895, KF875719, KF875915,* **KF875794**, *KF875896,* **KF875795**, *KF875721, KF875917,* **KF875797**, *KF875897, KF875916,* **KF875796**, *KF875722, KF875918, KF875898; Simpson Cliffs, MN USA,* 520363 (MIN), *FJ769163, KF875952,* **KF875827**, *KF875885, KF875710, KF875953,* **KF875828**, *KF875886, KF875712, KF875954,* **KF875829**, *KF875887, KF875713,* **KF875830**, *KF875714, KF875955,* **KF875831**, *KF875888, KF875715, KF875956, KF875716, KF875957,* **KF875832**; *Deer Creek, MN USA,* 787290 (MIN), *FJ769164, KF875924, KF875785, KF875919, KF875786, KF875700, KF875920, KF875852, KF875701, KF875925,* **KF875787**, *KF875853, KF875702, KF875703, KF875707, KF875921,* **KF875788**, *KF875854, KF875704, KF875922,* **KF875789**, *KF875855, KF875705, KF875706, KF875923,* **KF875790**, *KF875856, KF875708, KF875709; Glenora Cliffs, NY USA,* 456706 (MIN), *FJ769165, KF875723, KF875724, KF875725, KF875726, KF875727, KF875728, KF875729, KF875927, KF875928, KF875929,* **KF875798**, **KF875799**, **KF875800**, **KF875801**, **KF875802**, **KF875803**, *KF875857, KF875858, KF875859, KF875860, KF875861, KF875862;* **Rhodiola rhodantha (A.Gray) H.Jacobsen:** *Elwood Pass, CO USA,* 519453 (MIN) *KF875768, KF875769, KF875770, KF875771, KF875772, KF875949, KF875950, KF875951,* **KF875824**, **KF875825**, **KF875826**, *KF875882, KF875883, KF875884; Milner Pass, CO USA,* 519454 (MIN) *FJ769166,* **KF875823**; *Beartooth Highway WY USA,* 17997 (CHIC) *KF875773, KF875975,* **KF875848**, *KF875912; Continental Divide WY USA,* 17996 (CHIC) *KF875774, KF875972,* **KF875843**, *KF875911; Leigh Creek WY USA,* 17991 (CHIC) *KF875775, KF875971,* **KF875846**, *KF875909; Louis Lake WY USA,* 17992 (CHIC) *KF875973,* **KF875847**, *KF875899; Sheridan Creek WY USA,* 17994 (CHIC) *KF875777, KF875970,* **KF875845**, *KF875901; Powder River WY USA,* 17990 (CHIC) *KF875776, KF875974,* **KF875849**, *KF875900;* **Rhodiola rosea L.**; *Madison County, NY USA,* A18602 (NYS) *FJ769167, KF875759, KF875760, KF875761, KF875762, KF875960, KF875961,* **KF875837**, **KF875833**, *KF875891, KF875892, KF875893; Mount Horrid, VT USA,* 906586 (MIN) *KF875755, KF875756, KF875757, KF875758, KF875958, KF875959,* **KF875834**, **KF875835**, **KF875836**, *KF875889, KF875890*.