Evaluation of environmental factors effect on the genetic diversity, genetic structure and the potential distribution of *Rhododendron aureum* Georgi under changing climate

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Abstract

Understanding genetic variation and structure, adaptive genetic variation and its relationship with environmental factors is of great significance to understand how plants adapt to climate change and design effective conservation and management strategies. The objective of this study was to (I) investigate the genetic diversity and structure by AFLP markers in 36 populations of R. aureum from northeast China, (II) reveal the relative contribution of geographical and environmental impacts on the distribution and genetic differentiation of R. aureum; (III) identify outlier loci under selection and evaluate the association between outlier loci and environmental factors and (IV) exactly calculate development trend of population of R. aureum,as it is confronted with severe climate change and to provide information for designing effective conservation and management strategies. We found high genetic variation (I = 0.584) and differentiation among populations ($\Phi ST = 0.711$) and moderate levels of genetic diversity within populations of R. aureum. A significant relationship between genetic distance and environmental distance was identified, which suggested that the differentiation of different populations was the caused by environmental factors. Using BayeScan and Dfdist, 42 outlier loci identified and most of the outlier loci are associated with climate or relief factors, suggesting that these loci are linked to genes that are involved in the adaptability of R. aureum to environment. Species distribution models (SDM) showed that climate warming will cause a significant reduction of suitable area for R. aureum especially under the RCP 85 scenario. Our results help to understand the potential response of R. auruem to climatic changes, and provide new perspectives for R. auruem resource management and conservation strategies.

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Abstract: Understanding genetic variation and structure, adaptive genetic variation and its relationship with environmental factors is of great significance to understand how plants adapt to climate change and design effective conservation and management strategies. The objective of this study was to (I) investigate the genetic diversity and structure by AFLP markers in 36 populations of *R. aureum* from northeast China, (II) reveal the relative contribution of geographical and environmental impacts on the distribution and genetic differentiation of *R. aureum* ; (III) identify outlier loci under selection and evaluate the association between outlier loci and environmental factors and (IV) exactly calculate development trend of population of *R. aureum* ,as it is confronted with severe climate change and to provide information for designing effective conservation and management strategies. We found high genetic variation ($I = 0.584$) and differentiation among populations ($\Phi ST = 0.711$) and moderate levels of genetic diversity within populations of *R. aureum* . A significant relationship between genetic distance and environmental distance was identified, which suggested that the differentiation of different populations was the caused by environmental factors. Using BayeScan and Dfdist, 42 outlier loci identified and most of the outlier loci are associated with climate or relief factors, suggesting that these loci are linked to genes that are involved in the adaptability of *R. aureum* to environment. Species distribution models (SDM) showed that climate warming will cause a significant reduction of suitable area for *R. aureum* especially under the RCP 85 scenario. Our results help to understand the potential response of*R. auruem* to climatic changes, and provide new perspectives for *R. auruem*resource management and conservation strategies.

Keywords: Environmental factors, *Rhododendron aureum*Georgi, Genetic diversity, Genetic structure, Distribution, Climate change

1. Introduction

Genetic diversity is the basic requirement for species to long-term survive and adapt to environmental changes on an evolutionary time scale(E.E.K. Donald A. Falk, 2001; Frankham, 2005). Genetic structure is important as it can provide insights into the history of a population, and the current levels and distribution of genetic variation can influence the future success of populations(Erickson, Hamrick, and Kochert (2004). Under any combination of natural selection and random genetic drift, populations separated by geographic distance may diverge due to reduced gene flow and population connectivity (isolation by geographical distance, IBD)(Nosil & Rundle, 2012). Population divergence may still occur when reproductive isolation evolves between neighboring populations as a result of ecologically-based divergent selection in different environments (isolation by environment IBE)(I. J. Wang & Bradburd, 2014). Geographical processes may influence the population genetic structure at large spatial scales, while ecological processes may influence the population genetic structure at small spatial scales(Sacks, Brown, & Ernest, 2004).

Global climate change has become one of the major threats to biodiversity (M. B. Davis & Shaw, 2001; Camille Parmesan, 2006). Species may respond to global climate change by local adaptation(Margaret B. Davis, Shaw, & Etterson, 2008; C Parmesan, 2006), individual migration (Breshears, Huxman, Adams, Zou, & Davison, 2008; Lenoir, Gegout, Marquet, de Ruffray, & Brisse, 2008), range reduction(Thuiller, Lavorel, Araujo, Sykes, & Prentice, 2005) or a combination of these(Margaret B. Davis et al., 2008). Local adaptation has been found to be a conventional way of responding to climate change in various plant species. (Coop, Witonsky, Di Rienzo, & Pritchard, 2010; Gonzalez-Martinez, Krutovsky, & Neale, 2006; Hancock et al., 2011; Savolainen, Pyhäjärvi, & Knürr, 2007). Uncovering the genetic basis of local adaptations governed by natural selection is particularly important for understanding how plants adapt to their environment and respond to climate change. Reciprocal transplant experiments, quantitative trait locus (QTL) mapping and multiple-marker-based "neutrality" tests were used to investigate the local adaptations (Chartier, Pélozuelo, Buatois, Bessière, & Gibernau, 2013; Storz, 2005; Tanksley, 1993). However, because reciprocal transplant experiments and QTL mapping need to be based on phenotypic variation as a starting point, these approaches are generally restricted to a consideration of measurable traits that have already been implicated as candidates for different selection by independent lines of evidence, and they are unsuited to analyse adaptive genetic responses to climate change for the species which experience long juvenile phase in their life

history(Savolainen et al., 2007; Storz, 2005). Genome scans have been an approach to identify marker loci that are linked to selectively relevant target loci (outlier loci) through "genetic hitchhiking"(Luikart, England, Tallmon, Jordan, & Taberlet, 2003), and are widely used to detect the local adaptation of species to environmental conditions(Magdy, Werner, McDaniel, Goffinet, & Ros, 2016; T. Wang, Wang, Xia, & Su, 2016; A. H. Yang, Wei, Fritsch, & Yao, 2016b).Dfdist and BayeScan are two most commonly used methods. Dfdist builds an expected neutral distribution ofF_{ST}values under a classic symmetrical island model and loci potentially under positive selection can be identified if they exhibit unusually high F_{ST} deviations from neutral estimates(M. A. Beaumont & Balding, 2004; Mark A. Beaumont & Nichols, 1996); BayeScan evaluates population-specific F_{ST} values by considering different demographic histories and different amounts of genetic drift between populations(Foll & Gaggiotti, 2008). In this method,F_{ST} -based population genomic methods can be used to seek adaptive loci by scanning a lots of markers such as amplified fragment length polymorphism (AFLP) technique (Bensch & Akesson, 2005). The AFLP technique(Pieter Vos, 1995) has been commonly used to detect genetic diversity within and among populations, particularly in non-model organisms for which no prior genomic information is available. AFLP genome scans have been extensively employed to study plant populations, such as *Liriodendron chinense* (A. H. Yang, Wei, Fritsch, & Yao, 2016a), *Gentiana nivali* s(Bothwell et al., 2013),*Arabidopsis halleri* (Meyer, Vitalis, Saumitou-Laprade, & Castric, 2009), and *Sphaeralcea ambigua* (Shryock et al., 2015).

A major problem with genome scans is that they often detect false positives due to deviations from Hardy–Weinberg equilibrium and the assumption of the population structure model(L. Excoffier, Hofer, & Foll, 2009). Natural selection along environmental gradient or heterogeneity generates gradual changes (i.e. clinal variation) in allele frequencies at loci linked to selected genes(Manel, Poncet, Legendre, Gugerli, & Holderegger, 2010b). Consequently, outlier loci can potentially be detected by a closely association between allele frequencies and environmental parameters(Coop et al., 2010). The correlative approach need not consider the population structure and can be used to seek affirmation of outlier loci from the identification of candidate loci with genome scan methods(Joost et al., 2007; Nunes, Beaumont, Butlin, & Paulo, 2011; T. Wang et al., 2016; A. H. Yang et al., 2016b).

Natural population responses to global climate change by changing their geographical distribution, and species distribution models (SDM) have become increasingly popular tools for predicting the geographic ranges of species and have been important for predicting changes in distribution from past or future climatic events and for conservation(Hijmans & Graham, 2006; Kremen et al., 2008). Maxent, one of the most commonly used methods for inferring species distributions and environmental tolerances from occurrence data, allows users to fit models of arbitrary complexity(Warren & Seifert, 2011). Maxent calculates probability distributions based on incomplete information and does not require absence data, making it appropriate for modeling species distributions based on presence-only herbarium records(Merow, Smith, & Silander, 2013; Phillips SJ, 2006). During the past decades, many species' distribution have been studied by the Maxent, such as predicting habitat suitability of alien invasive weeds(Wan, Wang, Tan, & Yu, 2017), predicting the potential distribution of threatened medicinal plants *Fritillaria cirrhosa* and *Liliumnepalense*(Rana, Rana, Ghimire, Shrestha, & Ranjitkar, 2017), hindcasting the distributions of neotropical savanna tree species during the Last Glacial Maximum and Last Inter-Glacial(Bueno et al., 2017).

Rhododendron aureum Georgi (syn. *Rh. Chrysanthum* Pall.), the target plant species in this study, is a perennial evergreen creeping shrub with a large number of branched stems inhabiting alpine regions of Korea, China, Japan, and the Kamchatka peninsula. This plant can grow up to 1 m in height, and blooms from June to July in Korea with pale yellow flowers. It has been shown to always occupy the snowmelt gradient and especially to dominate in early exposed places(Kudo, 1992). In China, it grows mainly in the alpine tundra and the *Betula ermanii*population belts of Changbai Mountain, ranging from 1,000 to 2,506 m a.s.l.(Kudo, 1993). The *R. aureum* is one of the constructive and dominant species on the alpine tundra ecosystem, and it plays an important role in maintaining the ecological balance and preventing and controlling soil erosion.

Alpine environment is locally variable as small changes in altitude can lead to large changes in temperature, humidity, exposure, and other types of changes(Byars, Papst, & Hoffmann, 2007; Hovenden & Jkvander,

2004). With the global climate changing, in some alpine area, the increase in air temperature was more than twice as great as the increase in global mean air temperature during the 20th century(Bohm et al., 2001). Plant species are particularly vulnerable under the climate changing environment in alpine. Understanding the contemporary and historical ecological (climatic, geographical) factors shaping population genetic diversity is of great significance for studying molecular ecology, conservation biology and evolutionary biology (And & Hamrick, 1984; Holderegger, Buehler, Gugerli, & Manel, 2010).

In this study we adopted AFLP markers for characterizing the adaptive loci under selection using BayeScan and Dfdist, employed Multiple Linear Regression (MLR) to detect potential adaptive loci that are under selection from existing environmental factors, and using species distribution models (SDM) to predict potential distribution of *R. aureum* during the Last Glacial Maximum (LGM) and the future. The objective of this study was to (i) investigate the genetic variation and genetic structure of *R. aureum* ; (ii) reveal the relative contribution of geographical and environmental impacts on the distribution and genetic differentiation of *R. aureum* ; (iii) identify outlier loci under selection and assess the association between outlier loci and climate and (iv) exactly calculate development trend of population of *R. aureum* ,as it is confronted with severe climate change and to provide information for designing effective conservation and management strategies.

2. Materials and Methods

2.1. Study site and plant material

Changbai Mountain is generally recognized as the highest peak in northeast China and eastern Eurasia, with obvious mountain climate characteristics. This environment is locally variable as small changes in altitude can lead to large changes in temperature, humidity, exposure, and other types of changes(X. Yang & Wu, 1998). The alpine region on Changbai Mountain is the southern boundary of alpine tundra in east Eurasia. The varied topography, weather, soil and other natural conditions have created rich biodiversity and vertical zonal distribution of vegetation on Changbai Mountain, which has more than 2,277 species of plants and a notable richness of endemic species.

From 2012 to 2014, fresh leaves were collected from 461 individuals belong to 36 nature populations of *R. aureum* along an environmental transect which altitude range from 1200m to 2600m a.s.l., annual mean temperature range from 0.1 degC to -6.6 degC and annual precipitation range from 761 mm to 1096 mm**(Table 1, Figure C)** . These populations were scattered along peaks of Changbai Mountain (32 populations), Laobai Mountain (2 populations), and Wangtian'e Mountain (2 populations)**(Figure 1)** . In each of the population, a random sample from 7 to 17 individuals was obtained. In each locality, individual samples were taken from plants separated by at least 5 m (in order to avoid sampling clones) and dried directly in silica gel for transport back to Jilin University for subsequent DNA extraction.

Table 1. Summary of AFLP variation for 36 populations of

R. aureum.

Pop	GPS Coordinates(N/E)	GPS Coordinates(N/E)	Altitude (m)	N	PPL	I	H
N1	42.0292	128.0664	2600	12	41.871	0.228	0.154
N2	42.04018	128.0678	2300	8	58.797	0.329	0.224
N3	42.04202	128.0686	2200	14	31.403	0.153	0.100
N4	42.04575	128.0706	2100	14	22.272	0.123	0.083
N5	42.05552	128.0699	2000	10	37.862	0.208	0.142
N6	42.05935	128.063	1800	11	33.185	0.188	0.128
N7	42.09014	128.0687	1580	14	2.450	0.014	0.010
N8	42.13372	128.191	1200	14	18.708	0.103	0.070
NW1	41.99727	128.025	2462	14	52.116	0.293	0.200
NW2	42.0021	128.0249	2590	14	56.570	0.315	0.214
NW3	42.01297	128.0212	2161	9	30.958	0.166	0.112

Pop	GPS Coordinates(N/E)	GPS Coordinates(N/E)	Altitude (m)	N	PPL	I	H
NW4	42.0164	128.0252	2501	14	33.185	0.179	0.121
NW5	42.02153	128.0425	2198	7	32.071	0.176	0.119
NW6	42.02443	128.0402	2341	10	55.011	0.302	0.205
NW7	42.03347	128.0433	2431	14	41.425	0.212	0.140
NW8	42.03802	128.0485	2315	14	38.307	0.193	0.128
NW9	42.02957	128.0385	2500	7	39.421	0.225	0.153
NW10	42.043	128.0515	2122	14	36.526	0.189	0.126
W1	41.99007	128.0001	2223	14	35.857	0.196	0.133
W2	41.98912	128.0119	2138	14	32.517	0.163	0.109
W3	41.98713	128.0049	2014	14	30.067	0.154	0.102
W4	41.97795	128.0006	1872	14	33.408	0.171	0.114
S1	41.97879	128.0636	2535	14	48.998	0.257	0.172
S2	41.95675	128.0826	2099	14	34.744	0.179	0.12
S3	41.95501	128.0794	2083	14	37.639	0.198	0.133
S4	41.95376	128.0762	2030	14	50.78	0.278	0.188
S5	41.95178	128.0752	1945	14	35.412	0.177	0.118
S6	41.94115	128.0488	1814	14	30.735	0.181	0.125
NE1	42.0542	128.0714	2000	T	33.63	0.189	0.129
NE2	42.04927	128.0738	2100	9	29.176	0.172	0.118
NE3	42.04517	128.0749	2200	14	29.844	0.159	0.107
NE4	42.04015	128.0681	2300	14	29.399	0.156	0.105
L1	44.10465	128.0431	1698	14	39.866	0.218	0.147
L2	44.10325	128.0426	1701	14	34.967	0.185	0.125
WTE1	41.72863	127.902	2048	14	25.612	0.124	0.083
WTE2	41.72845	127.9011	2044	14	26.949	0.115	0.074
Total	–	–	–	461	99.777	0.584	0.402

N, population size; PPL, The percentage of polymorphic loci; H, Nei's (1973) gene diversity; I, Shannon's Information index

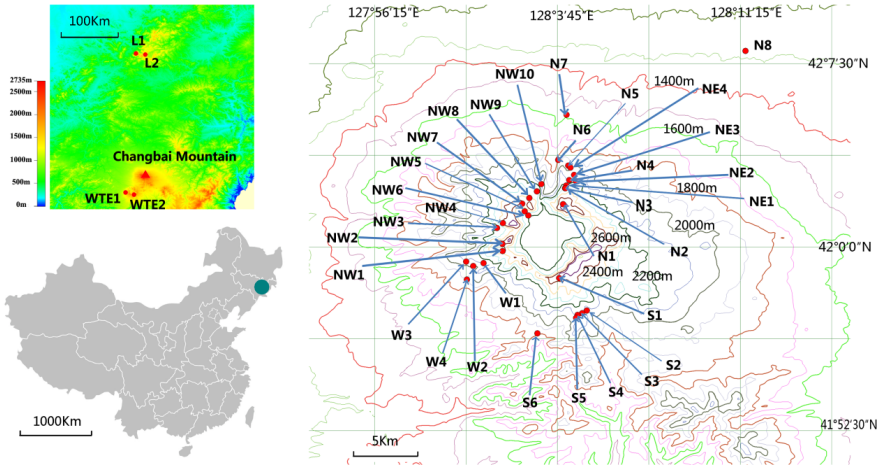

Figure 1. Distribution of 36 *R. aureum populations*sampled from China. We extracted the elevational information from DIVA-GIS Free Spatial Data (http://www.diva-gis.org/Data) and created the map by the

program Global Mapper version 13.00 (http://www.bluemarblegeo.com/products/global-mapper.php) and DIVA-GIS version 7.5.0.

2.2. DNA extraction, AFLP and marker scoring

The total DNA was extracted from the dried leaves by using a Plant Genomic DNA Kit (Bioteke Beijing Co. Ltd., Beijing, China). The DNA samples were diluted to 10 ng/μl and stored at -20°C until further analysis. AFLP marker was carried out according to the method of Vos et al. [30] with the following little modifications: the digestion–ligation reaction was performed in a 10 μl containing 1μl of 10 × T4 ligation buffer, 0.2 mM of ATP, 50 ng DNA, 1 U of T4 DNA ligase (Fermentas, Shenzhen, China), 1 U of *Eco* RI (Fermentas, Shenzhen, China), 1 U of *Mse* I (New England Biolabs, Beijing LTD), 1.0 μM of*MseI* -Adapter and 0.1 μM of *Eco* RI-Adapter and double-distilled water. The reaction was incubated at 37 °C for 8 h, 16 °C for 4 h, inactivated at 65 °C for 10 min, and stored at 4 °C. Pre-amplification was performed in a 25μl containing 2.5 μl of 10× PCR buffer and 0.5 U of Taq polymerase(Transgen Biotech Beijing Co. Ltd., China), 2.5μl of diluted digestion–ligation product, 0.2 μM of dNTPs, 0.3 μM each of primers with a single selective nucleotide. The pre–amplification conditions was as follows: pre-denatured at 94 °C for 5 min and 30 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 80 s, with a final extension for 10 min at 72 °C. The pre-amplification products were diluted 1 to 40 (v/v) with ddH_2O.

The selective amplification was essentially the same as that for pre-amplification except that 2 μL diluted pre-amplification product was used as template, and 2 μM *Eco* RI and *Mse* I selective primer were used. 10 pairs of primers were selected for selective amplification (**Table A**). The selective amplification reaction had two cycle sets: pre-denatured at 94 °C for 5 min, 13 cycles of 94 °C for 30 s, 65 °C (which was lowered 0.7 °C at each cycle) for 30 s and 72 °C for 60 s, followed by 18 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 80 s. After selective amplification, the products were added 25 μl of formamide loading buffer (98% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Then the products were denatured at 95 °C for 5 min and quickly cooled on ice, and separated on 6% denaturing polyacrylamide gel in 1× TBE buffer at 70 W for 4.5 h. Gels were stained according to the silver staining method(Bassam BJ, 1991).

2.3. Data analysis

2.3.1. Genetic Diversity and Genetic Structure

The AFLP bands were scored as present (1) or absent (0),and the AFLP band data were transferred to a binary (1/0) data matrix for further analysis. Shannon's information index (I) (RC, 1972), percentage of polymorphic loci (PPL), and genetic distance were estimated using the POPGENE v1.31 (Yeh F, 1997).Total genetic diversity (H_T) and mean genetic diversity within populations (H_S) were calculated using Nei's(Nei, 1973) genetic diversity statistics. A UPGMA tree based on genetic distance(Nei, 1978) was performed among different populations to identify their genetic relationships using NTsyspc v2.02 (Rohlf, 1997).

Population genetic structure was further assessed using model-based Bayesian assignment as implemented in STRUCTURE 2.3.4 software(Pritchard, Stephens, & Donnelly, 2000). Clustering of individuals was constructed without using the geographical origin of the samples as an informative prior. Analyses were based on an admixture ancestry model with correlated allele frequencies for a range of K genetic clusters from 1 to 36, with 20 replicates per K. The analyses were performed with a burn-in period and a run length of the Monte Carlo Markov chain (MCMC), of 10000 and 100000 iterations, respectively. The most likely number of genetic clusters (K) was estimated by the ΔK statistic (Evanno, Regnaut, & Goudet, 2005), using Structure Harvester (Earl & Vonholdt, 2012). Then CLUMPP 1.1.2(Jakobsson & Rosenberg, 2007) was used to align the 20 runs of the most representative K value and to compute the pairwise symmetric similarity coefficients (G) between pairs of runs, the average pairwise similarity (H) for the 20 replicates and the average probability of belonging to each cluster (Q). For K = 2, the full search method with 1000 replicates was used. A hierarchical analysis of molecular variance (AMOVA) was used to determine genetic differentiation (F_{ST}) within and among the groups. A nested AMOVA taking into consideration the two main genetic groups resulting from the Bayesian clustering with STRUCTURE (K = 2). using ARLEQUIN

version 3.5.1.2(L Excoffier & Lischer, 2010).

2.3.2. Outlier Detection

Two complementary methods were used to detect outlier loci of all populations of *R. aureum.* BayeScan version 2.1. identifies markers with unusually high or low levels of genetic differentiation as outliers that have signatures of diversifying or balancing selection, respectively (Foll & Gaggiotti, 2008). Specifically, selection is concluded from an AFLP marker if the marker-specific estimates ofF_{ST} are needed in addition to population-specific estimates to interpret observed patterns of differentiation in the dataset(Fischer, Foll, Excoffier, & Heckel, 2011). A threshold value for determining loci under selection was estimated according to Jeffreys' interpretation(Harris, 1961), i.e., $\log_{10}$ PO > 2.0 was regarded as adequate evidence for selection. A threshold of log10 PO > 2.0 was employed to reject the null hypothesis in each of the conducted tests. The analysis was performed with 20 pilot runs and a 50,000 step burn-in followed by 50,000 iterations and a thinning interval of 10 for the set of polymorphic AFLP markers. The false discovery rate (*FDR*) calculated the expected proportion of false positives for statistically significant results(Foll & Gaggiotti, 2008). We used the software Dfdist to simulate a null distribution of F_{ST} values under an island model, which was relative insensitive to demographic structure, population structure and mutation level. Simulations were conducted with a meanF_{ST}equal to the trimmed mean F_{ST} , which was calculated by excluding 30% of the most extremeF_{ST} values observed in the empirical dataset. We analysed the distributions of the F_{ST} values over all loci to null hypothesis of neutral evolution. Loci with a high or low F_{ST} value were taken as potentially under selection. In the study, we simulated the neutral distribution ofF_{ST} with 10,000 iterations at the 99.9% confidence intervals.

2.3.3. Environmental data and correlation analyses

To characterize environmental differences, BIOCLIM variables were obtained for each of the 36 sites by extrapolating climate data to the GPS coordinates for each population using DIVA-GIS software (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005; Hijmans, Guarino, Cruz, & Rojas, 2001). The BIOCLIM dataset includes 19 variables that describe monthly temperature and precipitation patterns for a spatial resolution of 1 km^2(*http://www.worldclim.org/*). The sampled area spanned all the known *R. aureum* range in China, which occurs primarily in the alpine, along an annual mean precipitation gradient from 761-1096 mm and annual mean temperature gradient from -6.6°C to 0.1°C. Elevation, slope, and topographic index were derived from a 90m digital elevation model. The data set is provided by International Scientific & Technical Data Mirror Site, Computer Network Information Center, Chinese Academy of Sciences (*http://www.gscloud.cn*). A principal component analysis (PCA) was applied to these environmental variables to examine possible correlations between eco-climatic variables and elevation and remove redundant variables (i.e. variables that were correlated at $|r| > 0.8$ and which were logically related). We first identified variables correlated to each retained axis, creating groups of variables. Within each group, we kept only one (or two) variables considered to be the most pertinent in terms of local adaptation in plants. Finally, eleven factors (Bio 1, annual mean temperature; Bio 2, mean diurnal range; Bio 3, Isothermality; Bio 4, temperature seasonality; Bio 5, max temperature of warmest month; Bio 9, mean temperature of driest quarter; Bio 16, precipitation of wettest quarter; Bio 17, precipitation of driest quarter; slp, slope; asp, aspect; tpi, topographic position index) were chosen as representative of environment factors.

Prior to subsequent analyses, environment data were $\log_{10}(x+1)$ transformed to improve normality and reduce heteroscedasticity. Dissimilarity matrices of Euclidean distances were calculated among normalized environment variables using R package. A matrix of geographic distances among sites was generated from GPS coordinates with the AFLP data in R software(Ehrich, 2006) and also $\log_{10}(x+1)$ transformed. The genetic distance matrices of *R. aureum* was calculated with PopGene(Yeh F, 1997) using the Jaccard dissimilarity method of the scored bands, were tested against the Euclidean distance of the environment variables while controlling of the geographic distance matrix. The Mantel test was performed using the default preset values and parameters (999 permutation steps) according to the software manual using R program "vegan" package. Multiple matrix regression with randomization (MMRR) is a novel and robust approach for estimating the independent effects of potential factors, and the analysis was implemented with 10,000 permutations

in R with the MMRR function script(Goslee & Urban, 2007; I. J. Wang, 2013; Wu, Yu, Wang, Li, & Xu, 2015).

Then, to detect relationships between allele frequencies and environmental variables, we applied Multiple Linear Regression (MLR)(Zulliger, Schnyder, & Gugerli, 2013) in R v.3.3.3 to determine potential adaptive loci that are under selection from existing environmental factors. For outlier loci identified by both Dfdist and BayeScan, we estimated their population pairwise frequencies of AFLP alleles at the 36 sites. We selected values of the eleven selected environmental factors from each population. We then regressed the allele frequencies of the retained outlier loci (dependent variables) on the selected environmental variables (explanatory variables; standardized) using the MLR model. Potential adaptive loci were identified as $R_{adj}{}^2 >$ 0.5. Univariate regressions were then applied to each variable individually to estimate its significance.

2.3.4. Species distribution model (SDM)

We used Maxent 3.3.3k to predict distribution changes for *R. aureum* as a result of climate changing. Maxent is a program for maximum entropy modelling of the geographical distributions of species; it combines presence-only data with ecological-climatic layers to predict suitable areas (Phillips SJ, 2006; Phillips & Dudik, 2008). For current distribution, we downscaled climate grids for the periods 1970–2000. In addition to sample locations in this study, we also collected the distribution records of *R. aureum* from the Chinese Virtual Herbarium (*http://www.cvh.ac.cn/*). After removing duplicate records, it remained a total of 42 records of*R. aureum* (**Table B**)that were used to establish the distribution model by using 19 bio-climatic data layers from the WorldClim database. Most of the default parameters of Maxent were applied to conduct SDM, except the following user-selected parameters: application of random seed and random test percentage of 70%, replicates of 10 and bootstrap as the replicated run type. The logistic output of Maxent consists of a grid map with each cell having an index of suitability between 0 and 1. Low values show that the conditions are unsuitable for the species to live, whereas high values show that the conditions are more suitable. Model predictions were visualized using DIVA-GIS(Hijmans et al., 2001).

To obtain the distribution of *R. aureum* at the Last Glacial Maximum, we projected correlation between current species-climate and the LGM using the Community Climate System Model (CCSM4) scaled down to a 2.5-arcmin resolution. We used the Hadley Global Environment Model 2 (HadGEM2-ES) as a general circulation model under two climate scenarios (IPCC-CMIP5 RCP 26/85) to ensure the accuracy of assessment. The RCP 85 scenario represents a higher predicted greenhouse gas emission than RCP 26.

3. Results

3.1 Patterns of AFLP variation and population structure 3.1.1. Subsubsection

The ten AFLP primer combinations generated 449 unambiguously scorable bands ranging from 1500 to 100 bp in 461 individuals from 36 natural populations. Of these fragments, 99.777% (448) were polymorphic. The overall Shannon's Information index (I) and Nei's genetic diversity (H) was 0.584 and 0.402 respectively.*R. aureum* showed high genetic diversity at the species level. Iranged from 0.014 to 0.329 and H ranged from 0.01 to 0.228 within population **(Table 1)** . The genetic diversity of most populations is at moderate level; but there is still a low genetic diversity of some populations, such as population N7.

Bayesian clustering analyses performed with the software STRUCTURE indicated that the most informative representation of overall genetic structure was achieved for $K = 2$, where K is the number of subpopulations. The southern slope populations (populations S1–S6) and the western populations on the U-shaped valley of the northern slope (populations NW7 and NW8) formed cluster 2, while populations NW9 and NW10 showed a high degree of membership to cluster 2. Populations L1 and L2, which are not on the prominent peak of Changbai Mountain and populations from the western slope region showed that, with exception of populations NW3 and NW4, individuals had a high level of admixture. The remaining populations displayed a high degree of membership to cluster 1

(Figure 2).

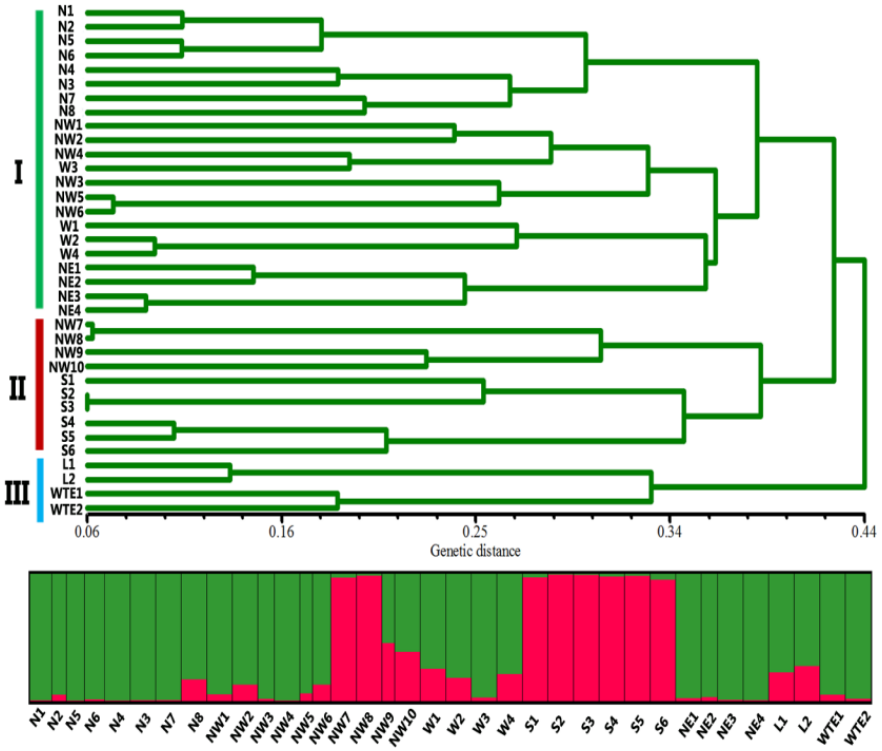

Figure 2. Phylogenetic relationships among 36 populations of*R. aureum* . inferred from AFLP data using a UPGMA tree (up) and with the STRUCTURE result at K = 2 (below).

The consensus UPGMA tree of populations contained three main groups **(Figure 2)** . Group I contained all the northern, western, and northeastern slope populations. Group II was composed of all the populations from the southern slope and some populations on the western end of the U-shaped valley (populations NW7–NW10). The remaining populations (populations L1, L2, WTE1, and WTE2), which do not occupy the peak of Changbai Mountain, clustered in group III.

An AMOVA **(Table 2)** attributed 68.87% of the overall genetic variation to the among-population component. A nested AMOVA that considered the two main genetic groups based on the Bayesian clustering analysis with the software STRUCTURE ($K = 2$) attributed 12.31% of the global variation to differences between the two clusters, 58. 78% to among-populations within clusters and 28.91% to within populations ($\Phi ST = 0.711$; both at $P < 0.0001$).

Table 2. AMOVAs for AFLP variation surveyed in a total of 36 populations of

R. aureum.

Structure analyzed and source of variation	d.f.
(a) Global analysis	**(a) Global analysis**
Among populations	35
Within populations	425
(b) Grouping following STRUCTURE clustering (K = 2)	**(b) Grouping following STRUCTURE clustering**
Among clusters	1
Among populations within clusters	34
Within populations	425

3.2.Correlations between genetic variation and environmental versus geographical factors

The Mantel test showed a significant correlation between genetic distance and environmental distance ($r = 0.4871$, $P = 0.001$), but between genetic distance and geographical distance was a non-significant correlation ($r = 0.0971$, $P = 0.028$). When geographical factors were controlled, a partial Mantel test also showed isolation by environmental distance ($r = 0.4797$, $P = 0.001$). Whereas environmental factors were controlled, we could not detect significant correlations between genetic differentiation and geographical distance ($r = 0.0118$, $P = 0.378$). The MMRR analysis indicated that the environment factors had large regression coefficient, whereas the effects of geographic distance were not significant (geographic distance: $\beta = 0.0094$, $P = 0.1075$; environment distance: $\beta = 0.2372$, $P = 0.0001$;**Table 3**).

Table 3. Results of the Mantel test, partial Mantel test and MMRR analyzing the correlation between geographical distances, environmental distances and *Nei's* genetic distance based on AFLP data.

	Mantel test	Mantel test	Mantel test	partial Mantel test	partial Mantel test	partial Mantel test	partia
	r	P value			r	P value	
Gen.Geo	0.0971	0.028	0.028	0.0118	0.0118	0.378	0.378
Gen.Env	**0.4871**	**0.001**	**0.001**	**0.4797**	**0.4797**	**0.001**	**0.001**

Regular letters refer to non-significant results and bold letters refer to significant correlations. Geo, geographical distance; Gen, genetic distance; Env, environmental distance.

3.3. Outlier analyses and MLR analysis

BayeScan determined 71 loci as outliers with a $\log_{10}$PO above 2, which is a threshold for adequate evidence for accepting a model under selection, corresponding to a posterior probability greater than 0.99 (**Figure 3A**). Using the Dfdist, we identified 126 adaptive loci at the 99.5% confidence intervals **(Figure 3B)** . 42 outlier loci were identified using two complementary analyses. The extremely strict significance criteria in the two approaches also assured the robustness of 42 outlier loci. Lastly, 21 potential loci under selection were verified by the MLR analysis with${R_{adj}}^2 > 0.5$ (**Table 4**). When we ran linear regressions using each environmental variable individually, all these eleven environmental variables were associated with the potential loci under selection and 30 loci were significantly (P$<$ 0.05) associated with at least one of the eleven selected environmental variables.

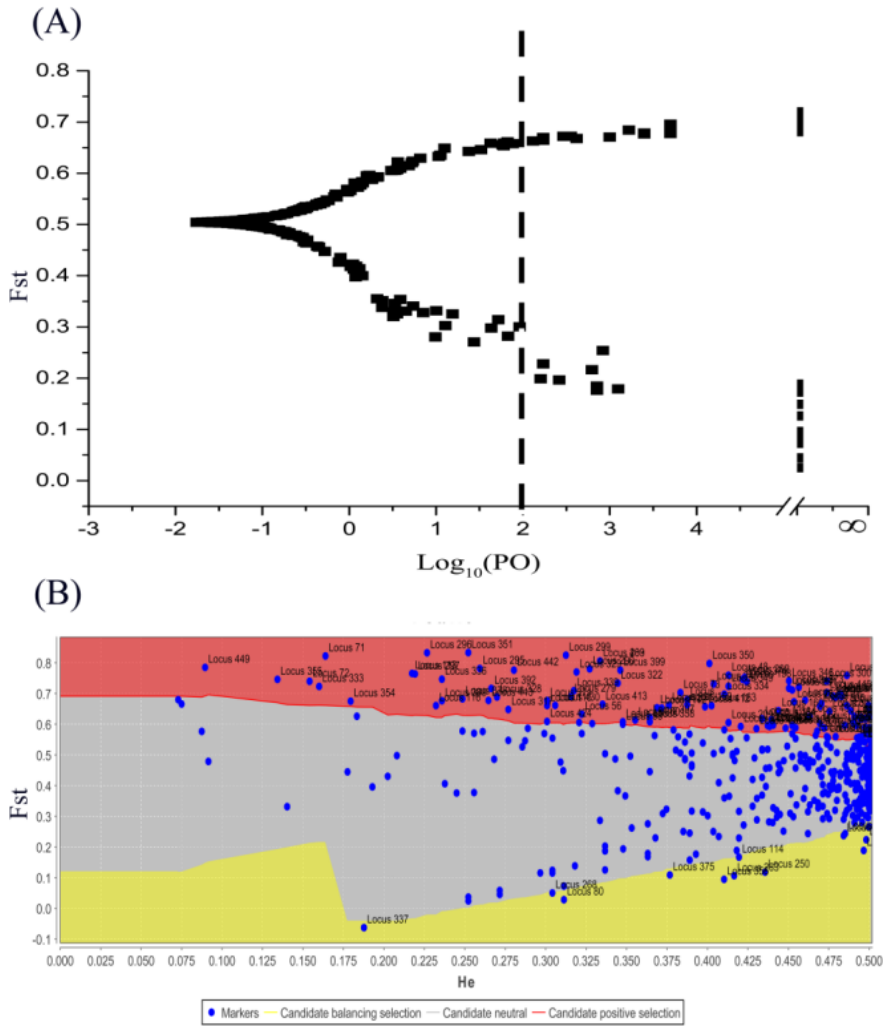

Figure 3. Outlier loci identified by BayeScan and Dfdist. (**A**) Plot of F_{ST} values and $\log_{10}$PO for 461 loci identified using BayeScan. Lines $\log_{10}$PO = 2 indicate "decisive" evidence for selection corresponding to a posterior probability of 0.99. Solid black dots greater than $\log_{10}$PO 2 represented outlier loci. (B) Outlier detection performed with Dfdist. Plot ofF_{ST} values of 461 loci in *R. aureum*populations was against heterozygosity.

Table 4. Results of outliers and environmental association analyses on AFLP loci of *R. aureum*

Outlier ID	Environmental variables R^2_{adj}	Significant environmental variables	Outlier ID	Environmental variables R^2
L48	**0.52**	BIO3,BIO9	L348	0.43
L73	**0.64**	BIO1, BIO3, BIO9	L349	0.39
L198	0.45	BIO2, slp	**L350**	**0.58**
L283	**0.52**	BIO2	**L352**	**0.53**
L287	**0.73**	BIO2, BIO16, BIO17, tpi	L373	0.30
L289	**0.61**	BIO1, BIO3	L374	0.36
L290	**0.54**	BIO1, BIO3	**L399**	**0.64**
L291	**0.50**	BIO1, BIO3	**L403**	**0.63**
L292	**0.51**	BIO3	**L404**	**0.55**

Outlier ID	Environmental variables R^2_{adj}	Significant environmental variables	Outlier ID	Environmental variables R^2
L293	**0.66**	BIO3	L405	0.49
L294	**0.57**	BIO3	L445	0.28
L298	**0.58**	BIO3,tpi	L53	0.34
L299	**0.65**	BIO2, BIO3, tpi	L59	0.42
L300	0.33	tpi	L80	0.40
L327	**0.64**	BIO1, BIO9, tpi, slp	L250	0.42
L332	0.33	–	L263	0.19
L334	**0.59**	tpi	L268	0.28
L335	**0.58**	BIO2, tpi, slp	**L304**	**0.62**
L344	0.33	–	L337	0.18
L345	0.41	–	L356	0.42
L346	0.47	BIO4, BIO16, asp	L375	0.28

slp, slope; tip, Topographic position index; asp, aspect

3.4. LGM, present and future distribution of R. aureum.

The average training AUC for ten replicate runs is 0.981, and the standard deviation is 0.035 which indicated an excellent predictive model performance. Minimum training presence logistic threshold was 0.15. The predicted distribution of*R. aureum* (**Figure 4B**) is consistent with the present distribution records including Changbai Mountain, Wangtiane Mountain, Laobai Mountain and North Koera, showing that the distribution of *R. aureum* is conditioned by environmental factors. The distribution of the LGM based on CCSM4 (**Figure 4A**) was substantially different from the present. The assessed distribution of *R. aureum* during the LGM was expanded to Korean Autonomous Prefecture of Yanbian and northern mountain of Korean peninsula. The predicted future and present distribution of*R. aureum* was considerably different in geographical range (**Figure 4C, 4D**). The major difference was that the predicted future suitable habitats showed a significant lessening in comparison with the present one. Only the peak of Changbai Mountain and some area in the North Korea would suitable for *R. aureum* under the rcp85 scenario. Loss of suitable habitats because of the climate changing indicated a drastically range contraction.

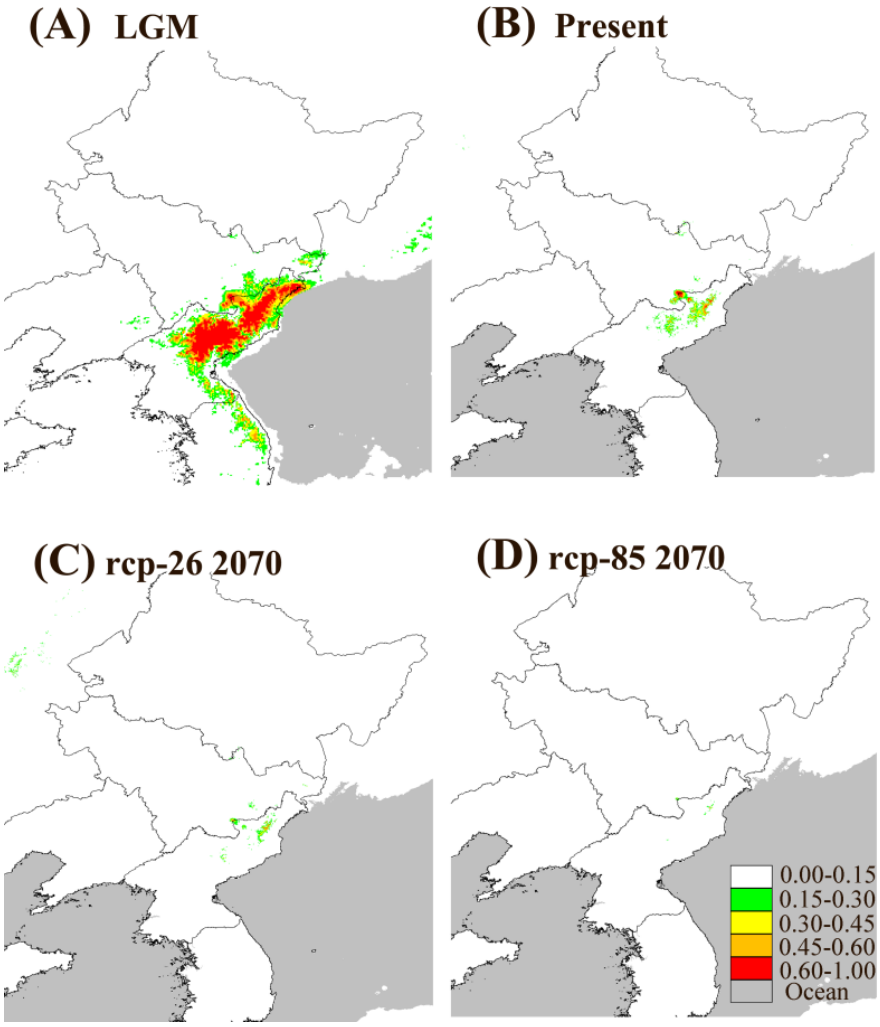

Figure 4. Potential distributions as the probability of occurrence for *R. aureum* . Suitability values indicate logistic probabilities ranging from 0–1, with increasingly darker shades of red with increasing habitat suitability. (A) Last Glacial Maximum (LGM) scenario; (B) present scenario; (C) RCP 26 scenario; (D) RCP 85 scenario (MAXENT v3.3.3k & Adobe Photoshop CS3).

4. Discussion

R. aureum exhibited a higher level of genetic diversity at the species level (I = 0.584, H = 0.402) than other Ericaceae species similarly researched with AFLP markers, such as *R. ledebourii, R. dauricum, R. sichotense* (Tikhonova, Polezhaeva, & Pimenova, 2012); and the high level of genetic diversity were in accordance with studies on other arctic and alpine species (H. A. PERSSON, 2001). High genetic diversity was also observed of *R. aureum* by RAPD and ISSR markers at the species level(Liu et al., 2012). Plant species with wide altitudinal ranges encounter different environmental conditions across the elevation gradient, which may lead to genetic variation as well as phenotypic variation among populations (Anna-Barbara Utelli, 1995; Forsman, 2014; Nicotra et al., 2015; Ohsawa & Ide, 2008). *R. aureum* is a long-lived, perennial, evergreen, dwarf shrub which altitude range from 1000m to 2600m in alpine regions. Along elevational gradients of alpine area, large changes in environmental factors, such as temperature, precipitation (**Figure C**), solar radiation, and wind, occur over short distances, resulting from obvious changes in the selection pressures of *R. aureum*individuals. Heterogeneous habitats strengthen disruptive selection to increase variation and diver-

gent selection pressures promote the evolution of traits adapted to their local environment, (Freeland, 2005). Divergent selection can promote genetic differentiation by reducing gene flow among sites with contrasting ecological conditions(Forester, Jones, & Joost, 2016). Results also showed that the genetic variability was even greater among populations (68.87%) but smaller within populations (31.13%), and there are high levels of differentiation among populations (Φ_{ST}= 0.689). Meanwhile, the high population differentiation could possibly accelerate local adaptation. Local adaptation and directional selection should have locus-specific effects of reducing genetic diversity within populations and increasing differentiation between populations (Magdy et al., 2016). Furthermore**,** long-lived perennial species with mixed breeding systems usually have relatively high genetic diversity (Nybom & Bartish, 2000). In the long-term evolutionary process, the high genetic variation held by *R. aureum*may have provided abundant genotypes for its adaptation to changing climatic conditions. There were some populations got the relatively lower genetic diversity than others. Population N7 inhabit on the low altitude in the coniferous forest which has a forest barrier from the others. Possible explanation for the low diversity found in the population is that small populations and habitat fragmentation are more susceptible pollen limitation, limited gene flow and genetic drift leading to loss of genetic diversity (Norman C. Ellstrand 1993; Vranckx, Jacquemyn, Muys, & Honnay, 2012).

Genetic divergence between populations is shaped by a combination of drift, migration, and selection, yielding patterns of isolation-by-distance (IBD) and isolation-by-environment (IBE)(Weber, Bradburd, Stuart, Stutz, & Bolnick, 2017). Some researches on population genetic structure discovered that IBD plays a more important role in intraspecific genetic differentiation than IBE(Mosca, González-Martinez, & Neale, 2014), however, IBE was implied to have a stronger effect than IBD on genetic structure in other plant taxa(Gray et al., 2014). A stronger effect of IBE versus IBD was found for the genetic differentiation of *R. aureum* . A Mantel test, partial Mantel test and MMRR analysis all supported the effect of isolation by environmental distance. In the cluster analysis, the fact that some geographically close populations are separated by larger genetic divergence than expected also proved the IBD is not the major driver of population divergence of *R. aureum* . The prominence of IBE suggests factors related to the environment play a greater role in divergence of *R. aureum*populations than geographical isolation.*R. aureum* lives in diversified habitats across its distribution region, and ecological landscape heterogeneity may influence gene flow and connectivity among populations that are adapted to different environments. Possible mechanisms responsible for IBE are selection pressures from climate and relief factors.

In identifying outlier loci or adaptive loci, we sought to determine how selection may play a role in shaping genetic differentiation and adaptation along sharp environmental clines. All 42 outlier loci identified by both BayeScan and Dfdist was undergoing putative diversifying selection and balancing selection (Figure 3). Most of the outlier associated with environmental predictors across the alpine environmental gradient (Table 4), suggesting these regions of the genome seem to be diverging and that climate may play a role. Most outliers were associated with temperatures related predictors (especially BIO1 and BIO3), probably due to the steep gradient in temperatures along our sampled region. In addition, many outliers were associated with precipitation and relief related environmental predictors, suggesting that precipitation and relief may also be exerting spatially divergent pressure on genetic. As expected, temperature, precipitation were estimated as the major driving factors influencing allele frequencies at outlier loci, consistent with other studies examining drivers of adaptive genetic divergence in plants(Manel, Poncet, Legendre, Gugerli, & Holderegger, 2010a; Yoder et al., 2014). Temperatures and precipitation factors are very important for plant growth, development, survival, reproduction and defense(Poncet et al., 2010). However, there are little researches has found the relief related factors influence the adaptive genetic divergence(Manel et al., 2010a). In this study, we found many outlier loci were related to the relief factors, such as 5 outlier loci were related to topographic position index (tpi), 4 outlier loci were related to aspect (asp), 2 outlier loci were related to slope (slp) with high values of $R_{adj}{}^2$. The relief has complex indirect effects on the combination of snow distribution and slope specific interception of radiation, and has the direct influence of exposure on microclimate during the growing season(Korner, 2003).

We used MAXENT to predict the distribution of*R. aureum* under LGM (Last Glacial Maximum), present and future climate conditions. MAXENT captured well a major portion of current distribution of *R. aureum*

. With the climate changing from the LGM to future, *R. aureum* decreased its future distribution range under a climatic warming scenario, especially under the RCP (Representative Concentration Pathways) 85 scenario which higher level greenhouse gases are emitted than RCP 26 in the years to come. We found the suitable distribution range of *R. aureum* would be reduced to the high altitude tundra area but would lose the low altitude area in Changbai Mountain. This is consistent with previous studies on other alpine area. Ecosystems at high latitudes and altitudes are particularly sensitive to climate change. Climate change is causing many species to shift their geographical ranges as reviewed in many researches (Bellard, Bertelsmeier, Leadley, Thuiller, & Courchamp, 2012; Dawson, Jackson, House, Prentice, & Mace, 2011). The abundance and dominance of shrub species have increased in alpine and subarctic tundra ecosystems in recent decades (Brandt, Haynes, Kuemmerle, Waller, & Radeloff, 2013; Myers-Smith et al., 2011; Myers-Smith et al., 2015; Sturm, Racine, & Tape, 2001; Sturm et al., 2005; Tape, Sturm, & Racine, 2006) and climate warming has been considered the dominant factor driving these range expansions of shrubs (Brandt et al., 2013; Li et al., 2016; Naito & Cairns, 2011; Walker et al., 2006; Yu, Luedeling, & Xu, 2010). As an effect of global warming, upward shifting of plant species in high mountain systems was predicted for the near future(Pauli, Gottfried, & Grabherr, 1996). Climate-induced range shifts and population declines are expected to increase the prevalence of population bottlenecks and reduce genetic diversity within and among species. Long-lived species are particularly vulnerable to climate changes because they experience longer generation times, lower population turnover rates and slower rates of evolution(Staudinger et al., 2012).

5. Conclusions

In summary, by using AFLP markers, landscape genetic, and species distribution modeling analysis together, we are able to identify many environmental factors that have influenced on the genetic diversity and genetic structure, and we can predict the potential distribution area of *R. aureum* . Our analyses revealed high genetic variation and differentiation among populations and moderate levels of genetic diversity within populations of *R. aureum* . A significant correlation between genetic distance and environmental distance was identified, which suggested that environmental factors were the primary cause of the population differentiation. 42 outlier loci were identified in 36 populations of *R. aureum* alone the environmental gradient and most of the outlier loci are associated with environmental factors, suggesting that these loci are linked to genes that are involved in the adaptability of *R. aureum* to environment. The SDM indicates that climate change drastically reduces the potential distribution range of*R. aureum* . An urgent area of future study is identification of genomic regions that are associated with environment factors by RAD-Seq(Hohenlohe, Catchen, & Cresko, 2012) and EST (expressed sequence tags). We should take measures to protect this species, such as translocate the populations or establish captive populations that would otherwise go extinct.

Author Contributions: Conceptualization, X.C; methodology, W.Z., Y.H., Y.Z. and L.L.; formal analysis, W.Z. and X.L.W.; data curation, W.Z. and J.N.L; writing—original draft preparation, W.Z.; writing—review and editing, X.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A. The primers used for AFLP analysis

Adaptors	**Adaptors**	**Adaptors**
	EcoRI-1 5'-CTCGTAGACTGCGTACC-3'	MseI-1 5'-GACGATGAGTCCTGAG-3'
	EcoRI2 5'-AATTGGTACGCAGTCTAC-3'	MseI-2 5'-TACTCAGGACTCAT-3'
Preamplification primers	**Preamplification primers**	**Preamplification primers**
	5'-GACTGCGTACCAATTCA-3'	5'-GATGAGTCCTGAGTAAC-3'
Selective amplification primers	**Selective amplification primers**	**Selective amplification primers**

Adaptors	**Adaptors**	**Adaptors**
AFLP-1	5'-GACTGCGTACCAATTCACC-3'	5'-GATGAGTCCTGAGTAACAA-3'
AFLP-2	5'-GACTGCGTACCAATTCACC-3'	5'-GATGAGTCCTGAGTAACTA-3'
AFLP-3	5'-GACTGCGTACCAATTCAGG-3'	5'-GATGAGTCCTGAGTAACAT-3'
AFLP-4	5'-GACTGCGTACCAATTCAGG-3'	5'-GATGAGTCCTGAGTAACAG-3'
AFLP-5	5'-GACTGCGTACCAATTCACA-3'	5'-GATGAGTCCTGAGTAACTA-3'
AFLP-6	5'-GACTGCGTACCAATTCACA-3'	5'-GATGAGTCCTGAGTAACTT-3'
AFLP-7	5'-GACTGCGTACCAATTCACT-3'	5'-GATGAGTCCTGAGTAACAA-3'
AFLP-8	5'-GACTGCGTACCAATTCACT-3'	5'-GATGAGTCCTGAGTAACAT-3'
AFLP-9	5'-GACTGCGTACCAATTCACT-3'	5'-GATGAGTCCTGAGTAACAC-3'
AFLP-10	5'-GACTGCGTACCAATTCACT-3'	5'-GATGAGTCCTGAGTAACTA-3'

Appendix B

Table B. The distribution records of *R. aureum*

Sites	Longitude/E	Latitude/N	Sites	Longitude/E	Latitude/N
1	137.8000	35.80000	22	128.0485	42.03802
2	142.8667	43.71667	23	128.0385	42.02957
3	142.8833	43.55000	24	128.0515	42.04300
4	142.8667	43.55000	25	128.0001	41.99007
5	142.8667	43.68333	26	128.0119	41.98912
6	138.3667	36.00000	27	128.0049	41.98713
7	128.0664	42.02920	28	128.0006	41.97795
8	128.0678	42.04018	29	128.0636	41.97879
9	128.0686	42.04202	30	128.0826	41.95675
10	128.0706	42.04575	31	128.0794	41.95501
11	128.0699	42.05552	32	128.0762	41.95376
12	128.0630	42.05935	33	128.0752	41.95178
13	128.0687	42.09014	34	128.0488	41.94115
14	128.1910	42.13372	35	128.0714	42.05420
15	128.0250	41.99727	36	128.0738	42.04927
16	128.0249	42.00210	37	128.0749	42.04517
17	128.0212	42.01297	38	128.0681	42.04015
18	128.0252	42.01640	39	128.0431	44.10465
19	128.0425	42.02153	40	128.0426	44.10325
20	128.0402	42.02443	41	127.9020	41.72863
21	128.0433	42.03347	42	127.9011	41.72845

Appendix C

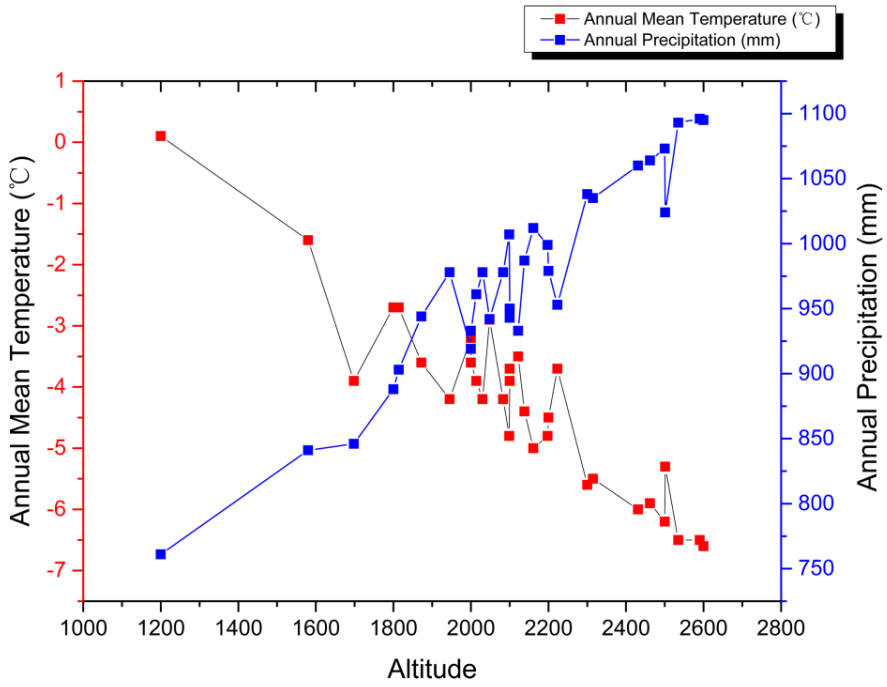

Figure C. The different annual mean temperature and annual precipitation along the altitude. The data was obtained from WordlClim database (http://worldclim.org/).

Data Accessibility Statement

We agree to deposit our data to a public repository. The data that support the findings of this study will be deposited in**Dryad** upon acceptance.

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