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Geometric Morphometric Analysis of Shape Variation in the Cone-Scales of *Pinus pumila* (Pall.) Regel (Pinaceae) in Kamchatka

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ABSTRACT

The purpose of this study was to test geometric morphometrics as a tool for analyzing phenotypic diversity of *Pinus pumila* (Pall.) Regel (Pinaceae). The study was conducted on the Kamchatka Peninsula. A sample of seed-bearing scales of mature cones was collected from 420 specimens in 14 coenopopulations, which represented the main ecotypes and associations of *P. pumila* in this region. Seven landmarks on one side of the cone-scales were chosen to characterize their shape. Patterns of shape variation within the sample were depicted using Generalized Procrustes Analysis (GPA). The results indicate that shape variables, relative deformations determined by methods of geometric morphometrics, can be used as reliable markers to estimate phenotypic diversity in *P. pumila*. Inter-population shape variability of *P. pumila* cone-scales is mainly determined by the latitudinal factor, which may date back to the expansion of *P. pumila* over Kamchatka in the postglacial age.

Keywords
geometric morphometrics, cone morphology, phenotypic diversity, Siberian dwarf pine

РЕЗЮМЕ

Ветрова В. П.
Геометрический морфометрический анализ изменчивости формы семенной чешуи *Pinus pumila* (Pall.) Regel (Pinaceae) на Камчатке

Цель настоящей работы – протестировать геометрическую морфометрию как инструмент для анализа фенотипической изменчивости кедрового стланика *P. pumila* (Pall.) Regel (Pinaceae). Исследование было проведено на полуострове Камчатка. Семенные чешуи зрелых шишек была собраны на 420 растениях в 14 ценопопуляциях, представляющих основные экотопы и сообщества *P. pumila* в данном районе. Семь меток на одной стороне чешуй было выбрано для описания их формы. Основные особенности вариации формы чешуй в исследованной выборке были выявлены с помощью прокрустова анализа данных. Было показано, что переменные формы – относительные деформации, определенные методами геометрической морфометрии могут использоваться в качестве надежных маркеров для оценки фенотипического разнообразия *P. pumila*. Межпопуляционная изменчивость формы семенных чешуй *P. pumila* определяется главным образом географической широтой, что может быть связано с историей его расселения на полуострове в послеледниковый период.

Ключевые слова
геометрическая морфометрия, морфология шишек, фенотипическое разнообразие, кедровый стланик

INTRODUCTION

Investigations of inherited morphological traits such as shape of seed-bearing scales are widely used in studies of phenotypic diversity and taxonomy of conifers (Pravdin 1964, Mamaev 1973, Popov 2005, Adrianova et al. 2011), and as phenotypic markers of population structure (Putenikhin et al. 2004, 2005). Traditional morphometric approach consists of applying multivariate statistical analyses to sets of morphological characters of cone-scales, including linear measurements of their length, width and ratios (Putenikhin et al. 2004, 2005). We used this approach before to analyze diversity within and among populations of *P. pumila* in Kamchatka (Vetrova & Savenkova 2010, Vetrova & Rostova 2012, Vetrova 2012).

Pinus pumila (Siberian dwarf pine) is a creeping stone pine with a geographical range over a vast area in Northeast Asia (Sochava 1986, Khomentovsky 2004). This species has highest genetic diversity in the *Pinaceae* family (Krutovsky et al. 1990, Goncharenko et al. 1991, Politov et al. 1992, Goncharenko et al. 1993, Krutovsky et al. 1994, Tani et al. 1996, Politov et al. 2004). *P. pumila* is a characteristic component of the coldest northern part of the Pacific monsoon region (Sochava 1986). We considered this part of *P. pumila*'s range as its optimum zone and used it to study phenotypic diversity. Owing to its geographic position and mountainous topography, Kamchatka provides a high variability of habitat sites and plant communities of *P. pumila* (Khomentovsky 2004, Neshataeva 2009). Several studies, which used traditional morphometrics methods, revealed a high level of *P. pumila* cone-scale polymorphism in Kamchatka (Vetrova & Savenkova 2010, Vetrova & Rostova 2012). Unfortunately, this approach cannot completely capture the geometry of the original object; besides, shape indices and size parameters of the scales are often correlated. Another method, partially related to classic morphometrics, is based on describing shape diversity of morphological structures using a number of discrete morphotypes (Zhivotovsky 1991). To reveal morphotypes of *P. pumila* cone-scales, four parameters were selected: ratio of width and length, angle at scale tip, length of scale tip, and the pattern of its decline (Vetrova 2012). Comparison of *P. pumila* samples based on occurrence frequencies of morphotypes enables a quantification of shape similarity of cone-scales. The disadvantage of this approach is that shape variables are continuous, so that morphotypes were defined by randomly dividing the variability rows into several intervals. Moreover, quantitative comparison of morphotypes cannot be defined and verification of reliability of the differences between them cannot be done.

For these reasons, we decided to describe shape diversity of *P. pumila* cone-scales using methods of geometric morphometrics, which have been actively developed since the 1990s (Bookstein 1991, Rohlf & Marcus 1993, Rohlf 1998, Adams et al. 2004). In contrast to traditional morphometrics, the geometric approach enables analysis of diverse shapes of morphological structures and conversion of different geometries. This method is based on an analysis of variation in the coordinates of landmarks that characterize the shape of morphological structures, using multivariate statistics. The standard procedure of morphometric analysis, referred to as a Generalized Procrustes Analysis (GPA) consists of four steps (Adams et al. 2004): 1) collect two- (or three-)dimensional coordinates of biologically definable landmarks and quantify raw data; 2) remove non-shape variation of landmarks by Procrustes superimposition of specimens with reference configuration; 3) perform multivariate analysis of the aligned coordinates; and 4) graphically visualize results in terms of configurations of the landmarks. A number of books (Bookstein 1991, Zelditch et al. 2004) and review articles (Pavlinov 2001, Pavlinov & Mikeshina 2002, Adams et al. 2004) provide a comprehensive theory and methods of geometric morphometrics and describe its application in biological research.

The purpose of this study was to test geometric morphometrics as a tool for analyzing *P. pumila* diversity. The tasks were: 1) to select and test morphological landmarks of *P. pumila* cone-scales; 2) to carry out multivariate analysis of shape variables in order to describe the diversity of *P. pumila* cone-scales' shape in a sample from 14 sites; 3) to estimate the influence of ecological and geographical factors on the diversity of *P. pumila* cone-scales; and 4) to evaluate differentiation of *P. pumila* populations in Kamchatka.

MATERIALS AND METHODS

The sample of *P. pumila* cones collected from 14 sites on the Kamchatka Peninsula is presented on Figure 1 and in Table 1. The sample represents all altitudinal ranges of *P. pumila* in the region (i.e., from marine terraces to highlands), three gradations of soil moisture regime (dry, fresh and wet), and three types of soil nutrient regime (poor, medium and rich). The sample from each population consisted of 3 to 5 cones collected from 30 randomly selected specimens. Three scales from the middle part of each cone were scanned and the most symmetrical scale with well pronounced features was selected as representative of a particular *P. pumila* specimen. The total sample contained scales from 420 specimens.

The data were processed and analyzed using Integrated Morphometrics Programs software (http:/life.bio.sunysb.edu/morph, http://www.canisius.edu/~sheets/morphsoft.html). The methods of geometric morphometrics used for the present analysis are also described in Zelditch et al. (2004).

An Epson Perfection V500 Photo scanner was used to digitize the scale images. Landmarks were placed and their coordinates determined using a TPSDig program (Rohlf 2010 a), while the editing of tps files was done using a tps Utility program (Rohlf 2010 b). Cone-scales are bilaterally symmetric structures, therefore, seven landmarks were positioned on one side of the scale (Figure 2, A): at the apex, at the base, at the greatest width, at three outline points (4, 5, and 6) placed at regular intervals using angular (20°) algorithm, with the last landmark 7 at the base of the scale tip. Because it was difficult to locate the position of landmark 7 on rhombic scales and those with weakly tapered tips, landmarks were first placed on scales that had a distinct bend of the outline at the base of the tapered scale tip. Subsequently, the average angle between the axis of symmetry and the radius from landmark 7 to the intersection point between the axis of symmetry and the greatest width line was calculated. The value of this angle (15°) was then used to determine the position of landmark 7 on rhombic and weakly tapered scales.

Initial coordinates of the landmarks were aligned using Procrustes superimposition (Fig. 2 B) in the CoordGen6 Coordinate Generating program (Sheets 2001) and, as a reference configuration, the mean structure for the samples of 420 scales was used. For graphical presentations of the statistical results, Sliding Baseline Registration (SBR), which prevents rotation of the symmetry axis of morphological structures, was applied (Fig. 2 C). Reconstruction of the whole scales was accomplished by duplicating the landmark coordinates and their reflection on midline of the scales (Fig. 2 D).

The main directions of the scale shape variation were revealed using the Principal Components Analysis (PCA) of partial warp scores including uniform shape deformations in the PCAGen6N program (Sheets 2001). Differences in shape of cone-scales between the 14 sampling sites were determined by paired comparison of the samples in the TwoGroup6h program (Sheets 2000). The distance between means of the samples was calculated as the sum of the squared distances between corresponding landmarks. To test the statistical significance of shape differences between the samples, Bootstrap resampling-based versions of Goodall's F-test was used for coordinates in the Procrustes superimposition, and Hotelling's T^2-test for coordinates in the SBR superimposition. Cluster analysis and

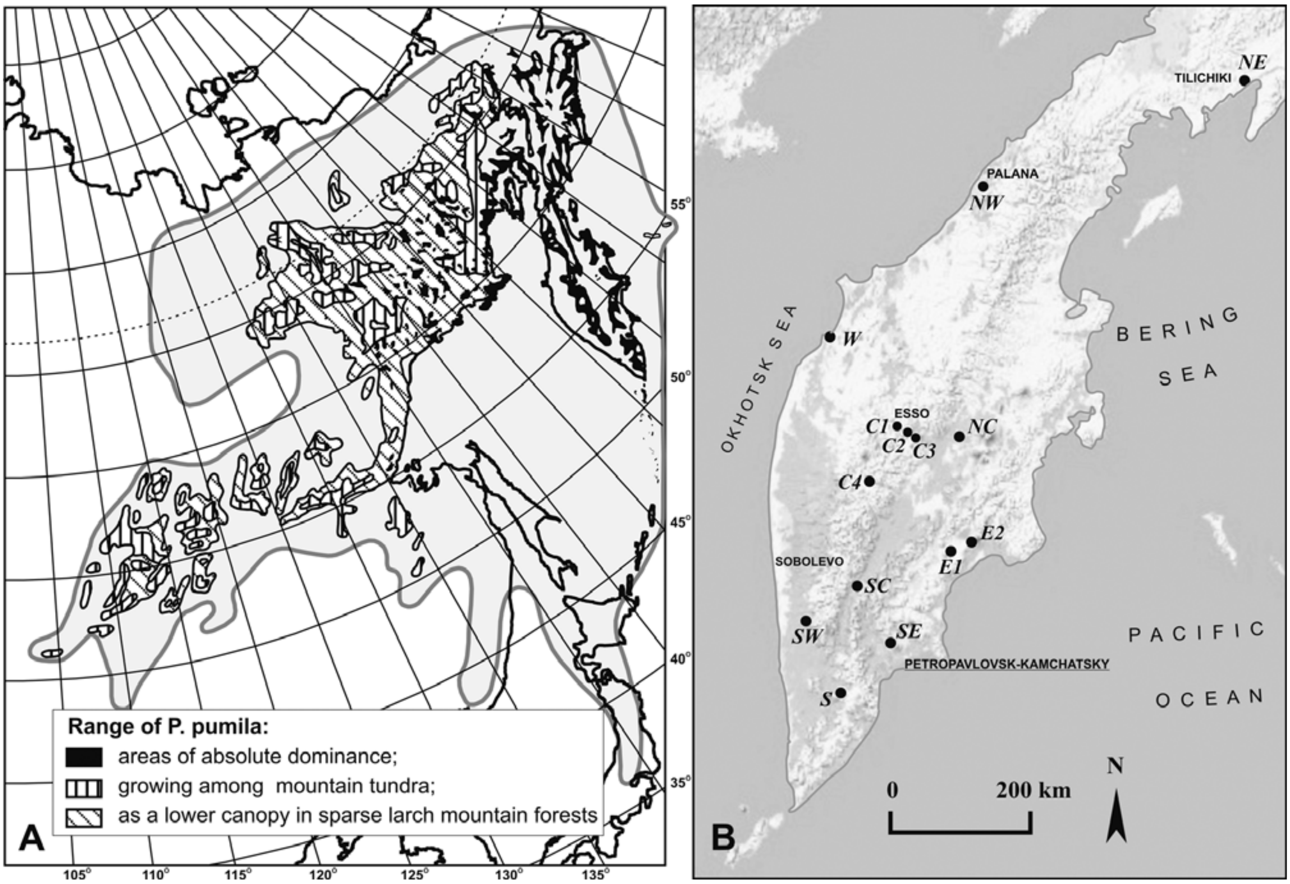

Figure 1 *Pinus pumila* range in North-East Asia (after Sochava 1986 and Khomentovsky 2004) (A) and the study area (B), showing locations of the 14 sampling sites on the Kamchatka Peninsula

multidimensional scaling of the distance matrix were performed using the Statistika 8 software package (Statsoft 2007). The analysis of the relationship between the shape of the scales and their centroid sizes and evaluation of the effects of environmental factors (altitude) on scale shape variation were conducted using multivariate regression analysis in Regress6 program of IMP software (Sheets 2001). Multivariate analysis of variance was used to estimate relationships between scale shape variables (PCA scores equivalent to relative deformations) and environmental factors represented by categorical variables of soil moisture regime and soil nutrient regime (ANOVA, Statistica 8). The effect of the geographical factor, including latitude, longitude and altitude, was determined using two-block partial least squares analysis of covariance in PLSMaker program of IMP software (Sheets 2003). Bootstrap-resampling test was used to assess the statistical significance of covariance between the blocks of shape variables and geographical data.

RESULTS

Shape variation of *P. pumila* cone-scales is expressed as scattered points characterizing coordinates of scale landmarks in the total sample relative to the landmarks of the reference configuration (Fig. 2). Shape diversity is mainly associated with localized deformations and is expressed as variations in the tapering and declining of the scale tip concordant with variations in the shape of the lateral margin of

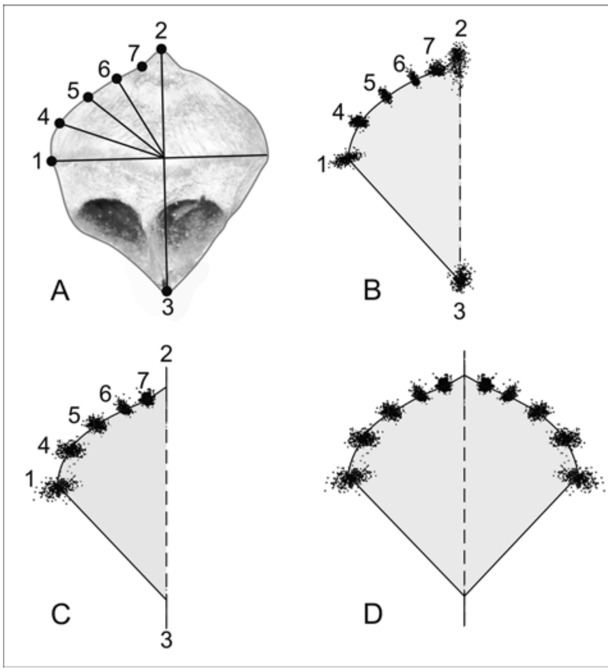

Figure 2 Shape variation of *P. pumila* cone-scales visualized by different superimpositions. A – seven landmarks recorded on the seed scale; B – Procrustes superimposition of the 420 seed-bearing scales sampled from the14 sites; C – Sliding Baseline Registration (SBR) of the shape coordinates of the sampled scales to the baseline formed by landmarks 2 and 3; D – reconstruction of the whole cone-scales by reflecting duplicated SBR-landmark coordinates across the midline

Table 1 Geographical and habitat characteristics of sampling sites of *P. pumila* on the Kamchatka Peninsula

Site location (ID)	Geographical coordinates	Plant community[1] [change to English names]	Elevation (m above sea level)	Soil moisture regime	Soil nutrient regime
North-eastern Kamchatka, Tilichiki (NE)	60°27'09" N 166°03'32" E	*Pinus pumila – Hylocomium splendens* (green moss) comm.	50	Fresh	Medium
North-western Kamchatka, Palana (NW)	59°03'45" N 160°00'17" E	*P. pumila – Hylocomium splendens* (green moss) – dwarf shrub comm.	30	Fresh	Poor
Western Kamchatka, Ust-Khairyuzovo (W)	57°01'50" N 156°38'19" E	*P. pumila – Calamagrostis langsdorffii* comm.	30	Fresh	Rich
North-central Kamchatka, Kozyrevsk, (NC)	55° 53'40" N 159°56'41" E	Open woodland *Larix cajanderi – Vaccinium vitis-idaea – Cladonia* comm.	130	Dry	Poor
Central Kamchatka, Esso (C1)	55°56'29" N 158°35'56" E	*P. pumila – Sphagnum* comm.	515	Wet	Poor
Central Kamchatka, Esso (C2)	55°55'32" N 158°43'25" E	*P. pumila – H. splendens* (green moss) – dwarf shrub comm.	500	Fresh	Poor
Central Kamchatka, Esso (C3)	55°54'28" N 158°45'58" E	*P. pumila – Rhododendron aureum* comm.	840	Fresh	Medium
Central Kamchatka, Aginskoe (C4)	55°16'30" N 157°49'42" E	*P. pumila – H. splendens* (green moss) – dwarf shrub comm.	792	Fresh	Poor
Eastern Kamchatka, Uzon (E1)	54°28'02" N 160°01'47" E	*P. pumila* – herbs – dwarf shrub comm.	667	Fresh	Medium
Eastern Kamchatka, Kronotskoe Lake (E2)	54°42'58" N 160°21'32" E	*P. pumila – Calamagrostis langsdorffii* comm.	400	Fresh	Rich
South-central Kamchatka (SC)	54°01'19" N 157°50'34" E	*P. pumila* – herbs – dwarf shrub comm.	505	Fresh	Medium
South-western Kamchatka (SW)	53°29'16" N 156°40'09" E	*P. pumila – Sphagnum* comm.	275	Wet	Poor
South-eastern Kamchatka, Avacha Volcano (SE)	53°16'02" N 158°44'08" E	*P. pumila* – sparse herb comm.	1007	Fresh	Medium
Southern Kamchatka, Tolmachevo Lake (S)	52°36'47" N 157°35'19" E	*P. pumila – H. splendens* (green moss) – *Cladonia* comm.	658	Dry	Poor

[1] Plant community type, soil moisture and soil nutrient regime according to V.Yu. Neshataeva (2009)

the scales. The most variable position is that of landmark 2, which characterizes the tapering and reflection of the scale tips. Considerable variation is also noticeable in the position of the landmarks corresponding to the diversity in the shape of tips, scale width and shape of its lateral margin.

The PCA of shape variables (including partial warp scores and the uniform component associated with uniform compression or extension of the scales) revealed four principal components, which account for 95 % of total explained variance in the position of landmarks. The visualization of these transformations in the vector form characterizes directions of scale shape variability corresponding to the maximum values of PCs scores for the sample, and Figure 3 is a graphic representation of these deformations as vectors relative to landmarks of the mean (reference) configuration. Scale deformations are shown as changes in the positions of the reference configuration landmarks due to SBR superimposition.

The first principal component comprises 45 % of explained variance of shape of cone-scales, with the shape varying from rhombic with an erect attenuate tip to broader with a declining tip (Fig. 3**)**. The second component accounts for 35 % of the variance and characterizes a change in scale shape from elongated with a declining apex to broad with an erect attenuate tip (Fig. 3). The third component explains 11 % of shape variance and reveals a change from round scales to broad scales with short mucronate tips (Fig. 3). The fourth component comprises 5 % of shape variation and describes a change from cordate to orbiculate-rhombic scales (Fig. 3).

The distribution of samples from the 14 sites on the factor plane shows a high degree of shape diversity of the cone-scales (Fig. 4 A). The ranges of relative deformations of the cone-scales in different samples determined by the PCA of partial warp scores overlap considerably, but significant differences in scale shape were recorded for 10

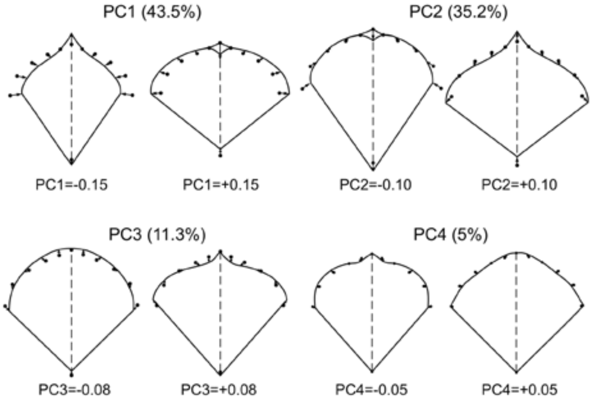

Figure 3 Principal components of shape variations in *P. pumila* cone-scales. Graphs represent transformation of the reference configuration corresponding to the maximum values of PC1-PC4 scores observed in 420 specimens of *P. pumila*. Arrows indicate change at each landmark of the reference (mean) configuration

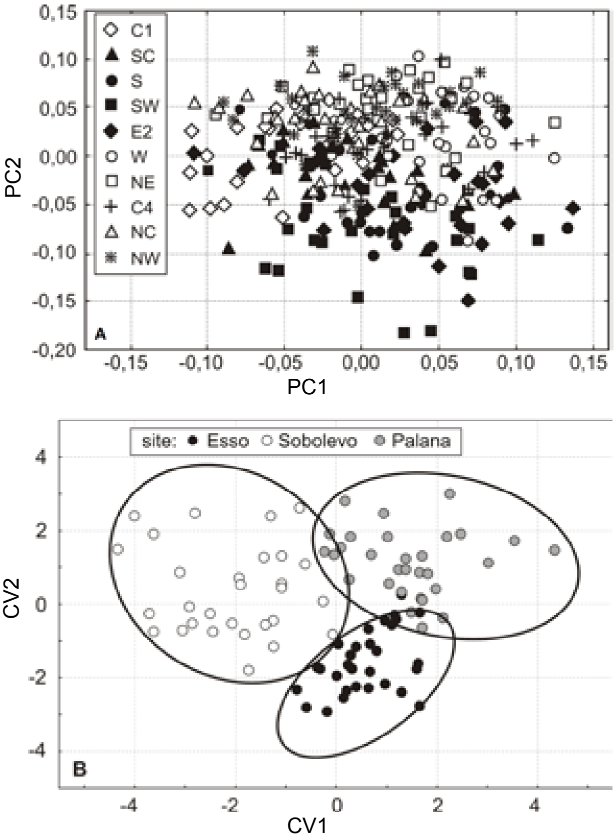

Figure 4 Ordination of the *P. pumila* samples from Principal Components Analysis and Canonical Variates Analysis. A – scatter plot showing ordination of 300 *P. pumila* specimens from 10 sampling sites on the first two principal components; B – scatter plot of canonical scores for 90 *P. pumila* specimens from three geographical areas

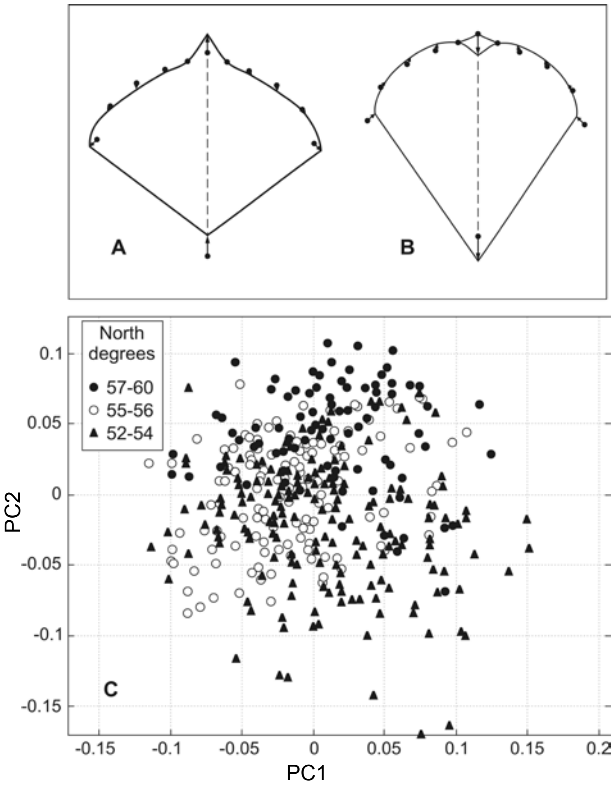

Figure 5 Geographical variation in shape of *P. pumila* cone-scales. Covariation between the shape of the cone-scales and geography are depicted as a transformation of the cone-scales configuration in the northern (A) and southern (B) directions. C: scatter plot showing ordination of 420 specimens of *P. pumila* on the first two principal components. Specimens coded to indicate latitude of their sampling sites

of the 14 sampling sites (Table 2). Partial Procrustes distances between pairs of mean shape of cone-scales from different sites varied from 0.009 to 0.138. The most significant differences were between *P. pumila* samples from three geographically distant areas (Fig. 4 B).

Multivariate analysis of variance showed that intrapopulation variation comprises at least 75 % of scale shape variation, while inter-population variation accounts for 25 %. Results of multivariate regression analysis suggest that shape is not related to centroid size of the scales. Altitude has an insignificant effect on scale shape variation, and the correlation coefficient of shape variables for this factor is 0.20. Further, multivariate analysis of variance did not reveal any statistically significant relationships between relative deformations of the cone-scales and edaphic variables of soil moisture and soil nutrient regime.

Partial least squares analysis extracts two dimensions of covariance between scale shape and geography which explain 88 % and 12 % of covariance between the these two data sets. The loadings of the first geographic variable

Table 2 Matrix of distances between samples of *P. pumila* cone-scales from 14 sites, using landmark coordinates in Procrustes superimposition (upper right side of the table) and landmark coordinates in SBR-superimposition (lower left side of the table). Shaded bold case indicates distances between samples which have a not significant difference between means at P≥0.95. Site locations, geographical and ecological characteristics are shown in Table 1

Site	C1	C2	C3	SE	E1	NC	C4	SC	S	SW	E2	W	NE	NW
C1	0	**0.018**	0.025	0.024	0.022	0.043	0.075	0.048	0.072	0.096	0.086	0.093	0.074	0.070
C2	**0.019**	0	**0.020**	**0.017**	0.022	0.040	0.068	0.034	0.058	0.084	0.073	0.088	0.074	0.072
C3	0.028	0.022	0	**0.012**	**0.008**	0.029	0.058	0.038	0.057	0.093	0.069	0.073	0.057	0.053
SE	0.028	**0.019**	**0.012**	0	**0.015**	0.032	0.058	0.029	0.049	0.085	0.064	0.073	0.061	0.060
E1	0.027	0.025	**0.009**	**0.016**	0	0.028	0.062	0.044	0.061	0.098	0.075	0.075	0.057	0.052
NC	0.060	0.061	0.044	0.047	0.040	0	0.044	0.064	0.076	0.109	0.074	0.067	0.038	0.038
C4	0.094	0.088	0.074	0.075	0.075	0.047	0	0.052	0.056	0.082	0.044	0.039	0.043	0.060
SC	0.052	0.037	0.039	0.031	0.046	0.064	0.073	0	0.029	0.058	0.042	0.072	0.073	0.078
S	0.081	0.066	0.062	0.055	0.067	0.076	0.075	0.034	0	0.064	0.029	0.061	0.076	0.082
SW	0.098	0.085	0.093	0.086	0.110	0.109	0.098	0.058	0.066	0	0.055	0.109	0.117	0.130
E2	0.098	0.084	0.077	0.072	0.082	0.074	0.054	0.052	0.036	0.065	0	0.059	0.077	0.088
W	0.116	0.110	0.073	0.092	0.092	0.067	0.041	0.092	0.078	0.125	0.065	0	0.047	0.035
NE	0.091	0.091	0.072	0.076	0.070	0.038	0.043	0.087	0.091	0.128	0.083	0.047	0	0.026
NW	0.086	0.087	0.066	0.073	0.063	0.039	0.060	0.090	0.093	0.138	0.092	0.037	0.026	0

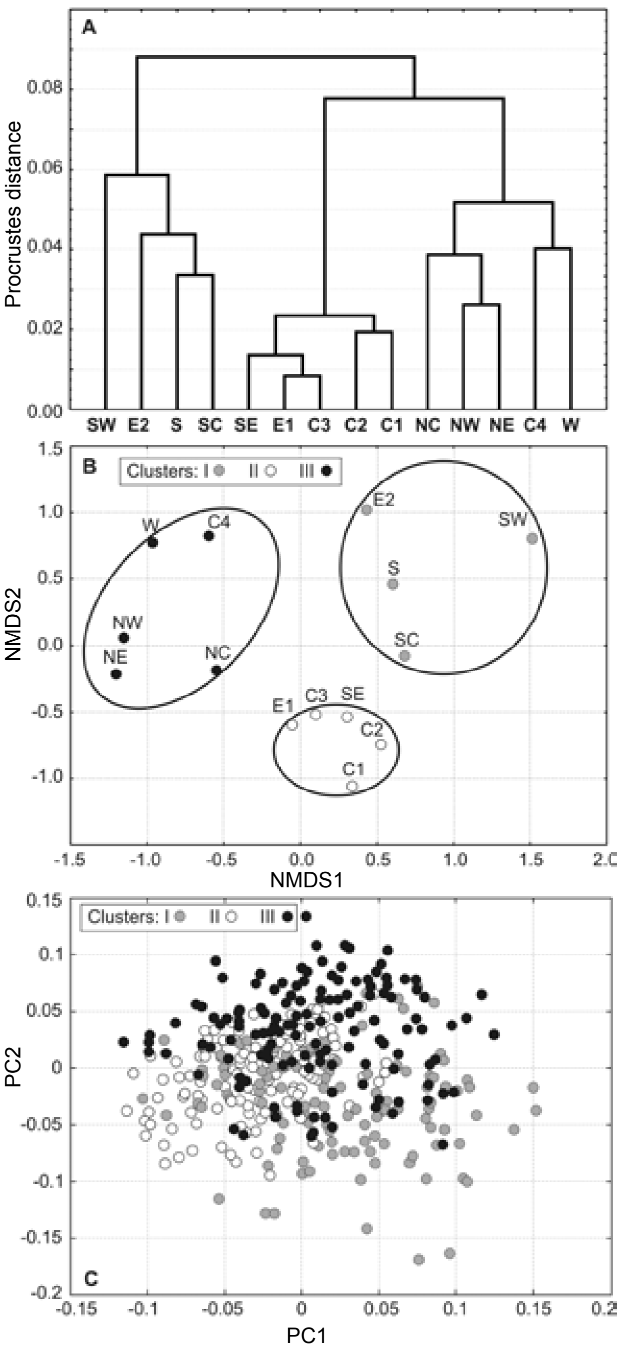

Figure 6 Differentiation of *P. pumila* populations in Kamchatka. A – tree diagram of 14 *P. pumila* coenopopulations derived from weighted pair group average clustering matrix of Procrustes distances between the samples of the cone-scales, B – Ordination of 14 *P. pumila* populations from non-metric multidimensional scaling matrix of Procrustes distances between the samples of the cone-scales. C – scatter plot showing ordination of the 420 specimens of *P. pumila* on the first two principal components. Specimens coded to indicate cluster of their sampling sites

suggest a latitudinal factor, with the factor loading for the latitude of 0.85, compared to 0.52 for the longitude. The correlation of 0.48 between the blocks of shape and geographic variables is also statistically significant. However, only 12 % of covariance between scale shape and geographical factor is related to altitude.

Geographic variation in scale shape is expressed as broader scales with erect tapered tip in northern populations (Fig. 5 A), compared to the southern populations in which round scales with uncinate tip prevail (Fig. 5 B). These differences are reflected in the ordination of northern and southern populations on the plane of principal components of scale shape variation (Fig. 5 C). Samples from central Kamchatka occupy an intermediate position on the factor plane.

Cluster analysis and multidimensional scaling matrix of the Procrustes distances between samples of the cone-scales divided the 14 sites into three clusters (Fig. 6 A). The first cluster includes two samples from southern Kamchatka (S, SW), one eastern sample (E2) and a sample from the south of the Central Kamchatka Depression (SC). The second cluster is composed of three samples from central Kamchatka (C1, C2, C3) and two samples from south-eastern Kamchatka (SE, E1). The third cluster includes three northern samples (NE, NW, W) and a sample from the north of the Central Kamchatka Depression (NC). The least significant differences are observed between geographically close samples from central Kamchatka and two samples from south-eastern Kamchatka (Figure 6, B, cluster II). Samples in the northern and southern groups are considerably differentiated (Fig. 6 B, clusters I and III), and their phenotypic distances in scale shape are an order of magnitude greater than those of the central Kamchatka group.

DISCUSSION

We conclude that this differentiation between *P. pumila* samples corresponds to its expansion during the postglacial age. Inter-population shape variability of *P. pumila* cone-scales is mainly determined by the latitudinal factor. *P. pumila* could have thus been disseminated from forest refugia (Central Kamchatka Depression) in both northern and southern directions. Sample differentiation within clusters may be caused by local adaptation and selective factors of microevolution.

This study shows that geometric morphometrics is an effective tool for analyzing *Pinus pumila* diversity. The fact that scale variables are not related to scale size or edaphic and topographic conditions suggests that they can be used as reliable quantitative characters for phenotypic diversity of *P. pumila*. The seven landmarks chosen for the analyses appear to provide a relatively comprehensive coverage of the form of *P. pumila* cone-scales and may probably be used for analyzing diversity of other stone pines with similar morphological structures of cone-scales. However, for a more complete description of shape diversity, asymmetry of cone-scales should also be considered. Geometric morphometrics can be used to define discrete phenotypes of cone-scales and analyze variability and diversity of conifer populations.

Our future studies will be aimed at investigating phenotypic variability of *P. pumila* in other parts of its range and at examining covariation between shape variables of cone-scales and molecular-genetic markers.

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