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ABOUT SPECIES INDEPENDENCE OF *POLYSTICHUM SUBTRIPTERON* TZVEL.

I.A. Kreshchenok[1], T.A. Sinitsyna[2], M.G. Kutsev[2], S.V. Smirnov[2], A.I. Shmakov[2]

[1] *Amur Branch of Botanical Garden-Institute, Far East Branch, Russian Academy of Sciences, Laboratory of Introduction*

[2] *Altai State University, Lenina str., 61, Barnaul, 656049, Russia; Email: ssbgbot@mail.ru*

The article discusses the species independence of *Polystichum subtripteron* Tzvel. The spore structure and size of 2 closely related species – *P. subtripteron* and *P. tripteron* were studied. Morphological differences of fronds fit into the overall range of variation; there are no differences in the spore external structure. DNA fragment analysis (ISSR) of 19 specimens of both species was conducted. Fragment analysis revealed no significant differences between *P. tripteron* and *P. subtripteron* samples. All differences are probably due to genetic isolation of populations, and the island populations are closer together. On the basis of the research we conclude that in the Far East one species *Polystichum tripteron* grows, and *P. subtripteron* can be considered its synonym.

Key words: *Polystichum subtripteron, Polystichum tripteron, fronds, spores, distribution, ISSR, DNA.*

INTRODUCTION

Polystichum Roth is the one of largest genera of ferns, consisting of 200 species all over the Earth (Shmakov, 2011). The greatest species diversity of the genus is in the regions of East Asia, where over 100 species spread on the territory from Japan to the Himalayas.

In the Russian Far East 6 species of *Polystichum* are distributed (Tzvelev, 1991), among them 2 close species – *Polystichum subtripteron* Tzvel. and *P. tripteron* (Kunze) C. Presl.

N. Tzvelev described *P. subtripteron* (Christmas fern almost tripartite) in 1989 on the samples from surroundings of Vladivostok city (Russia). Type: "Far East, Primorsky Krai, in the wood, near the city of Vladivostok, 10 VIII 1901, Herb. Russian flora No. 1350, N. Palchevskiy" (LE) (Tzvelev, 1989). As the main distinctive feature of this species from close *P. tripteron* author gave the ratio of length of middle part to the length of lateral parts of the frond.

Other morphological characters of these species are similar. These taxa have different geographical distribution. It is specified that *P. tripteron* grow on island territories (Sakhalin, southern Kuril Islands, Japan Islands), and *P. subtripteron* is distributed in continental part of the south of Far East and Manchuria (the south of Primorsky Krai, south-west of China, Korea) (Tzvelev, 1991; Shmakov, 2009a, 2009b, 2011). But there is no data about distribution of *P. subtripteron* on the territory of China (Clavis Plantarium…, 1995; Kung Hsianshiu, 2001) and Korea (Lee, 1993; Ferns and Fern Allies…, 2005; Kim, Sun, 2007) in the floristic summaries of China and Korea, only about *P. tripteron*. In connection with ambiguous interpretation of the taxa by botanists the necessity of more detailed taxonomic study of *P. subtripteron* and *P. tripteron* has been ripe.

MATERIALS AND METHODS

Variability of morphological parameters – the ratio of length of middle part to the length of lateral parts of the frond – was examined by line method in cm. The measurements were carried out at the herbarium specimens of *P. subtripteron* and *P. tripteron* from funds of Biology and Soil Institute, FEB RAS

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(VLA), South-Siberian Botanical Garden of the Altai State University (ALTB), Central Siberian Botanical Garden (NS, NSK), as well as on living plants from natural populations in Primorsky Krai (near Vladivostok) and Hirosima (Japan, S. Smirnov). The study of ultrasculpture of spore surface was conducted with a scanning electron microscope JSM-6390LV JEOL (Japan), shot in the secondary electron mode (the SEI), in the Analytical Center of the mineralogical and geochemical studies IGIP FEB RAS (Research Analyst – T. B Makeyeva).

The investigated samples:

Polystichum tripteron – Kuriles, Kunashir Island, slope of marine terrace, 4 km south-west from vil. Nazarovo, on pine forest edge, among of tall herbage, massively. 14.08.1985. V. Yu. Barkalov (VLA, № 04465, DNA 1381). *Polystichum subtripteron* – Primorsky Krai, near Vladivostok city, deciduous forest. 25.07.2010, Kreshchenok I. A. (DNA 1380).

Material for fragment analysis of DNA has been represented by herbarium material and leaves dried using silica gel.

We studied 19 samples of the genus *Polystichum* (1373, 1374, 1377, 1378, 1382, 1383, 1384, 1376, 1381, 1375, 1380, 1636–1647) from Japan (7 samples), Kunashir Island (2) and Vladivostok (10).

DNA was isolated using the Diamond DNA kit (LLC "ABT", Russia), in accordance with the manufacturer's instructions. Amplification was performed in 25 μl of a reaction mixture comprising 2 μl of DNA sample, 2.5 μl of 10x buffer and 25mM $MgCl_2$, 2 μl of 10mM primer, 1 μl of 5mM dNTPs, and 0.2 μl of Taq DNA polymerase, on a thermocycler MyCycler (Bio-Rad, USA), using SSR program: 95.0 ºC for 3 min. [95.0 ºC for 20 sec., 58.0 ºC for 30 sec., 72.0 ºC for 1 min. 30 sec.] $_{x1}$, [95.0 ºC for 20 sec., 56.0 ºC for 30 sec., 72.0 ºC for 1 min. 30 sec.] $_{x1}$, [95.0 ºC for 20 sec., 54.0 ºC for 30 sec., 72.0 ºC for 1 min. 30 sec.] $_{x1}$, [95.0 ºC for 20 sec., 50.0 ºC for 30 sec., 72.0 ºC for 1 min. 30 sec.] $_{x37}$, 72.0 ºC for 8 min., 4.0 ºC to the end.

Of the 15 primers, participated in the selection the most polymorphic primers were 17898B, 17899B and HB12, which we used for further analysis: 17898B – CACACACACACAGT, 17899B – CACACACACACAGG, HB 12 – CACCACCACGC (Kutsev, 2009). DNA fragments were separated by 1.5 % agarose electrophoresis gel. Then, the gel was stained with ethidium bromide and photographed under UV light. The matrix has been formed to phenetic analysis in Microsoft Excel program based on the presence (1) or absence (0) of fragments of the same length as determined by applying the ruler to each sample on gel electrophoretic pictures. The resulting matrix of 83 features for the 19 samples has been analyzed in the programs for phenetic analysis – NTSYS-pc and Structure v2.3.4.

RESULTS AND DISCUSSION

Analysis of the herbarium and living specimens of *P. subtripteron* and *P. tripteron* has shown that diagnostic character of the ratio of length of middle part to the length of lateral parts of the frond has a wide range of variation (Fig. 1). In *P. subtripteron* the values of this character lies in the range from 1.7 to 5. The most samples have the correlation value from 1.9 to 3. *P. tripteron* has the wider range of this character: from 2 to 9.8, but often have values 2.6–4.4, and the area of maximum character "overlap" lies in the range from 2 to 3.6.

Often the fronds of these species are asymmetric, and difference of the length of right and left lateral frond portions may be up to 3 cm (usually up to 1.5 cm), which greatly affects the value of the ratio of length frond portions. For example, when the length of the middle portion is 8.5 cm, and lateral portions – 2.3 cm and 3.3 cm, the length ratios in the first case will be 3.6 (it corresponds *P. tripteron*), while in the second case – 2.6 (it corresponds *P. subtripteron*). Image analysis of herbarium specimens of Chinese Virtual Herbarium, which unites the leading herbaria of China, has shown that plants with elongated as well as with short lateral parts of fronds grow on territory of continental Asia (Figs 2, 3).

These data show that the character of the ratio of length of middle to lateral frond portions is not clear diagnostic character and it is not enough for distinguishing *P. subtripteron* as separate species. Broad modification variability of length middle and lateral frond portions in individuals of island population is, probably, result of some climate conditions effect.

Spores of *P. subtripteron* bean-like, spore boundary smooth. Perispore large-folded, slightly peelable; folds friable, volumetric. Perispore surface not grainy, with multiple nipple-shaped excrescences and abundant small perforations (Fig. 4).

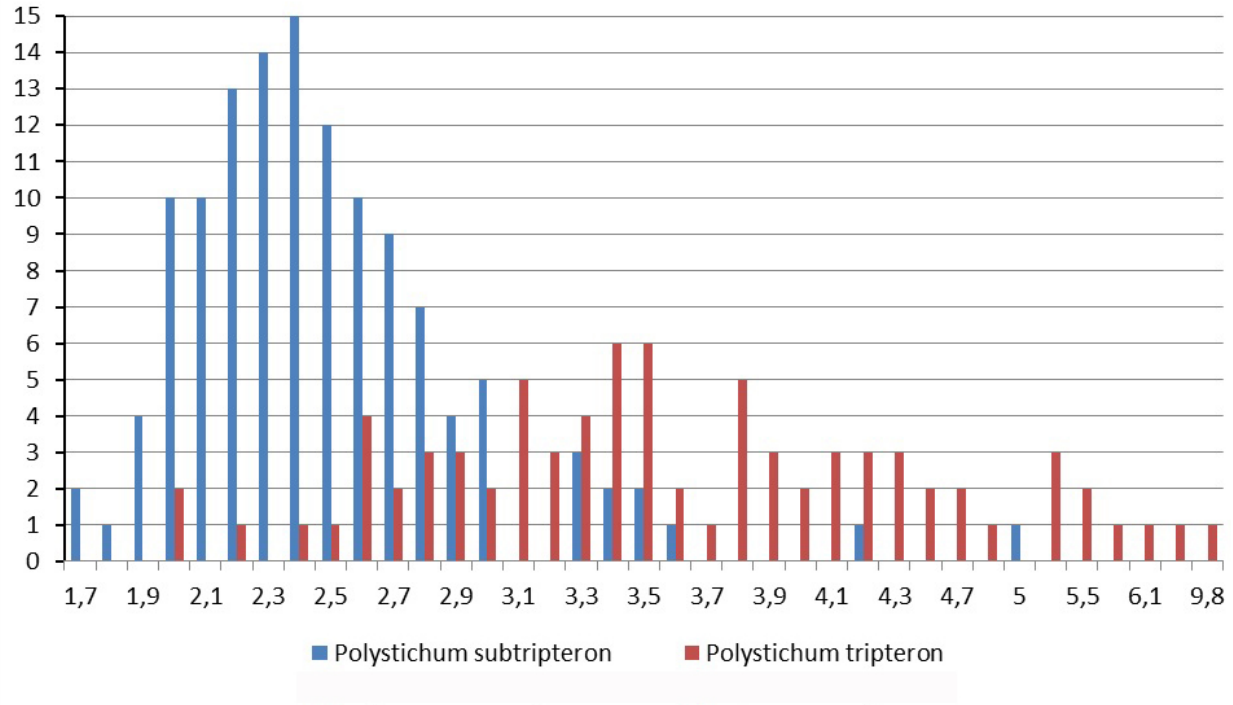

Figure 1. Variability of the ratio of length of middle to lateral frond portions of *Polystichum tripteron* and *P. subtripteron*.

Figure 2. *Polystichum tripteron* from China with short lateral parts.

Figure 3. *Polystichum tripteron* from China with elongated lateral parts.

Spores of *P. tripteron* bean-like, with smooth boundary. Perispore has large folds, lightly damaged and peeled off. Perispore surface not grainy, with multiple nipple-shaped excrescences, has many small perforations (Fig. 5).

Comparison of the morphology and ultrasculpture of spores of *Polystichum tripteron* and *P. subtripteron* has showed that the spores of both taxa have the same shape and smooth contour. Morphometric parameters of spores of these species are almost identical (Table 2). Perispore is of one type, with the same surface and perforation character. On the basis of this analysis, we have resulted that the spores of studied taxa are identical and do not have a clear diagnostic differences.

Table 2. Spore morphometric parameters of *Polystichum subtripteron* and *P. tripteron*

Species	Spore length (µm)	Polar diameter (µm)	Laesura length (µm)	Laesura width (µm)	Perispore thickness (nm)	Length of spore with perispore (µm)	Polar diameter with perispore (µm)	Width of perispore fold (µm)
Polystichum subtripteron	38,4±2	29,2±3	22,2±3	1,08±0,3	303±5	42,2±7	38,07±5	6,92±1
Polystichum tripteron	40,1±3	30,2±1	24,4±3	1,1±0,2	297,3±6	49,4±5	42,07±6	6,02±0,8

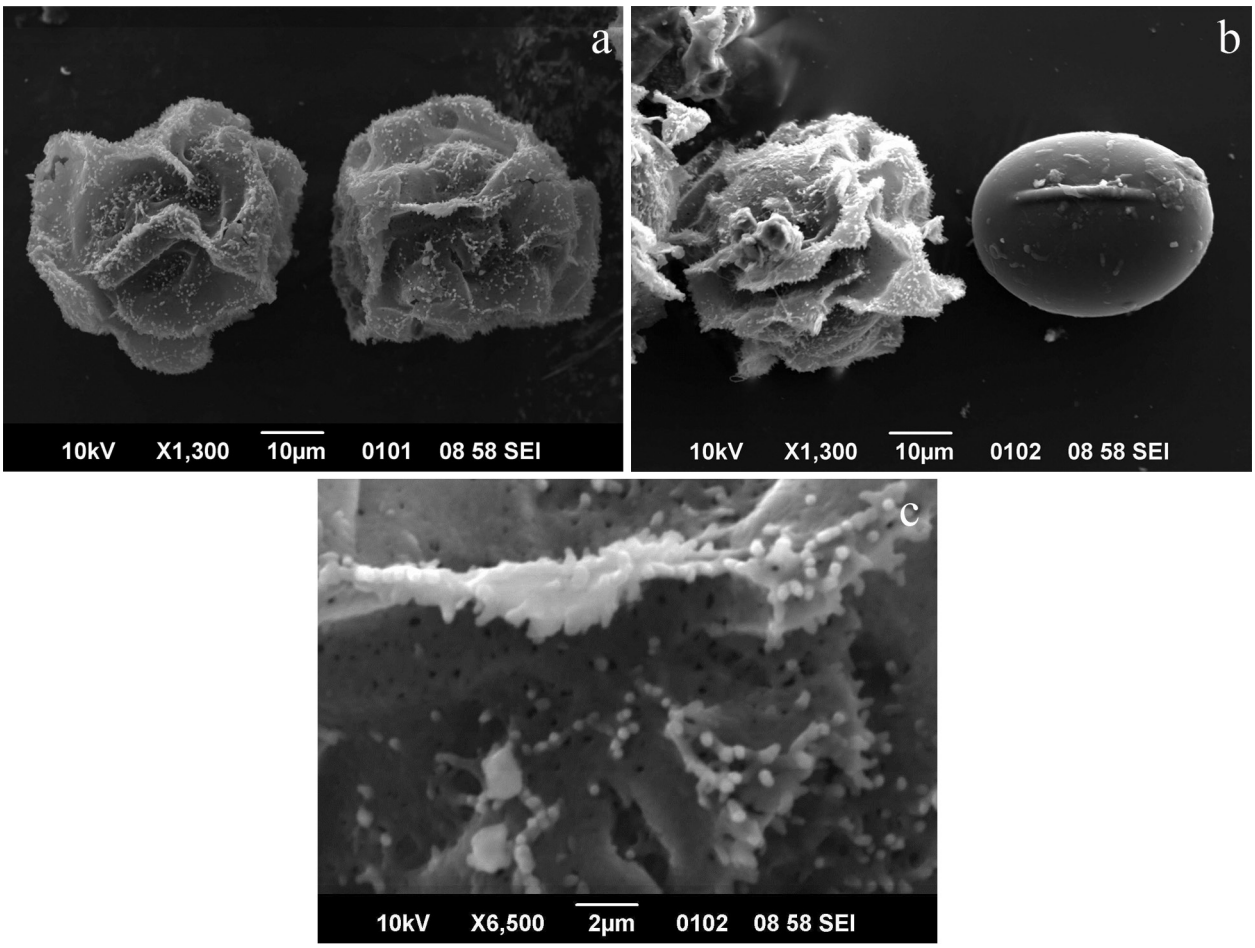

Figure 4. Morphological structure of spore of *Polystichum subtripteron*: a, b – external structure of spore, c – perispore structure.

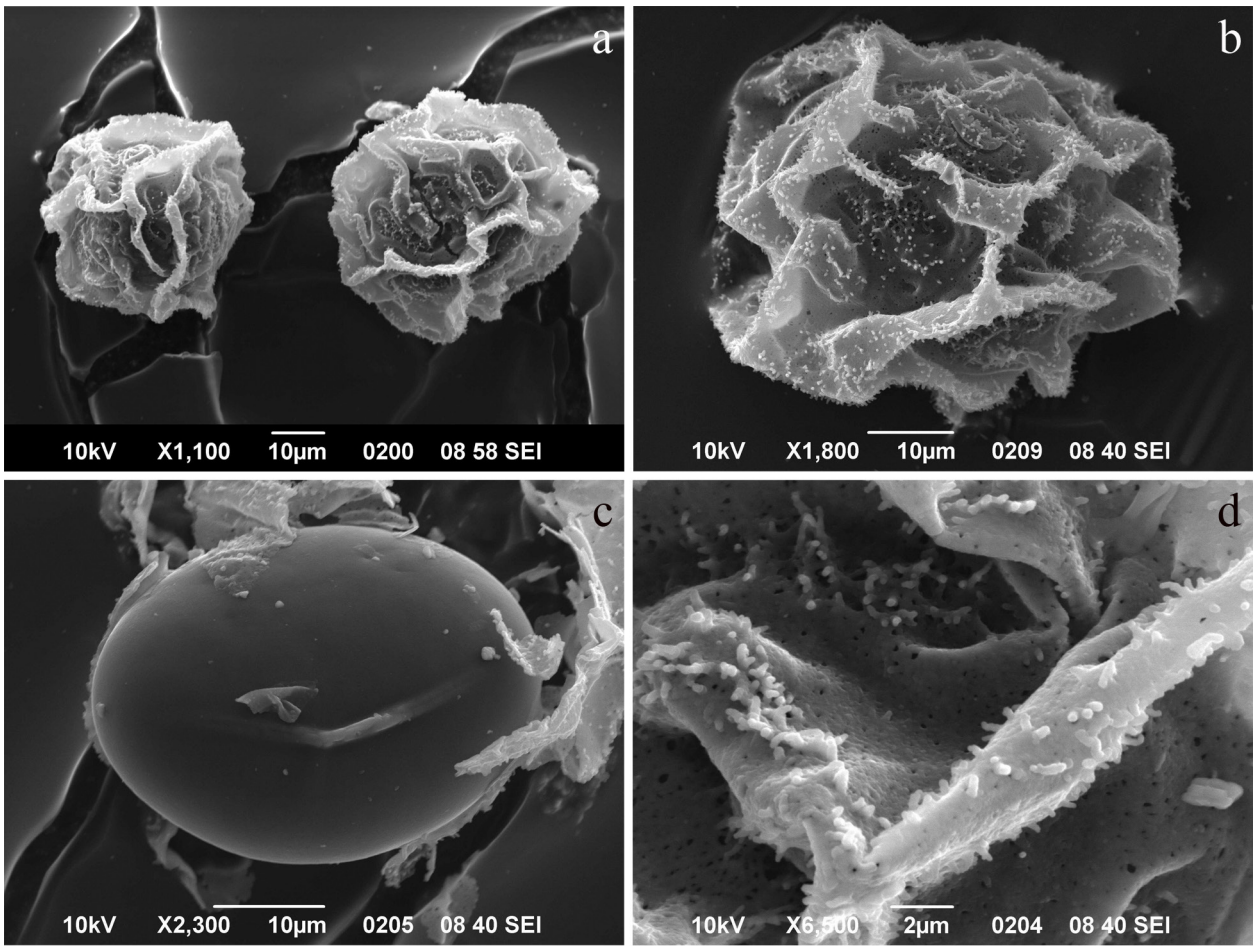

Figure 5. Morphological structure of spore of *Polystichum tripteron*: a, b, c – external structure of spore, d – perispore structure.

The areas of distribution of these species are close. N. Tzvelev (1991) noted, that *Polystichum tripteron* grew in Sakhalin Island, the Kuril Islands and Japan, and *P. subtripteron* he pointed to the southern mainland Asia (South of the Russian Far East, northeastern China, Korea). Japanese Sea is considered a natural boundary between the areas of the two species.

To clarify the taxonomic position of *P. subtripteron* we have used the DNA analysis of Inter-Simple Sequence Repeats (ISSR), which has good reproducibility and has been used successfully to identify interspecific and intraspecific genetic variation, species and population identification etc. (Kutsev, 2009). 83 polymorphic ISSR-loci have been identified for 19 samples. Cluster analysis of character matrix in the program NTSYS-pc using method of Neighbor-Joining (NJ) with M. Nei coefficient (Nei, 1972) has allowed reconstructing cladogram of the samples similarity.

Fragment analysis has revealed that 2 *P. tripteron* samples from Kunashir Island get to clade of Vladivostok samples of *P. subtripteron* (Fig. 6A). *P. tripteron* samples from Japan (1–7) form a separate clade. But analysis of the matrix by the Bayesian probabilities in Structure program, using for clusterization the sample on the basis of the data of the individual genotypes (Pritchard et al, 2000; Falush et al, 2003), has not allowed significantly dividing all the samples into groups for K from 1 to 5 (Fig. 6B). Thus, molecular genetic analysis has revealed no significant differences between the samples of *P. tripteron* and *P. subtripteron*, too. All differences are apparently associated with the genetic isolation of populations, and the island populations are closer together.

CONCLUSIONS

On the basis of the research it can be argued that in the Far East one species *Polystichum tripteron* grows, and *P. subtripteron* can be considered its synonym. Morphological differences of fronds fit into

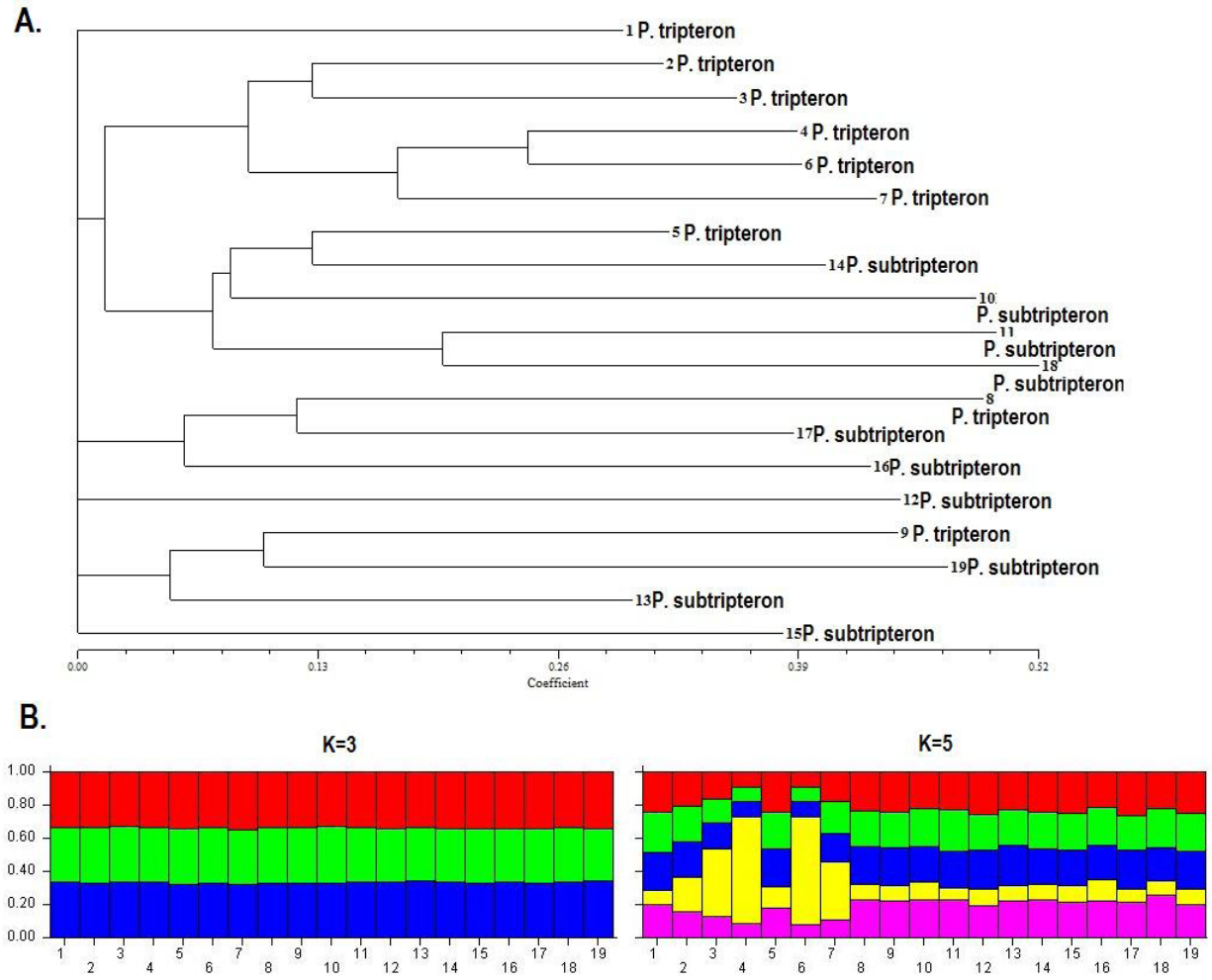

Figure 6. Clasterisation of *Polystichum* samples based on ISSR-markers: 1–7 – *P. tripteron*, Japan; 8,9 – *P. tripteron,* Kunashir Island; 10–19 – *P. subtripteron*, Vladivostok.

the total range of variation, there are no differences in the external structure of the spores. The fragment analysis has revealed that *P. subtripteron* samples from Vladivostok surroundings get to one clade with *P. tripteron* from Kunashir Island that shows their genetic propinquity; most likely, they are different populations of a single species *P. tripteron*.

The area of distribution of *P. tripteron* spreads to the southern part of the Russian Far East, Sakhalin Island, the Kuril Islands, northeast China, Korea, and Japan.

There is the species synonymy below.

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