SHORT COMMUNICATIONS
КРАТКИЕ СООБЩЕНИЯ

AN INTEGRATED APPROACH TO CONSERVATION OF RARE FERN SPECIES USING THE EXAMPLE OF *POLYSTICHUM CRASPEDOSORUM* (DRYOPTERIDACEAE) IN AMUR REGION, FAR EAST OF RUSSIA

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Currently changes in the earth's vegetative cover have reached a planetary scale. The danger of extinction of species and ecosystems has become increasingly significant. Active measures must be undertaken to restore particular plant species and populations in order to minimise an ongoing loss of species. *Polystichum craspedosorum* is a cliff-dwelling fern, an East-Asian relict species whose northern range includes the Russian Far East. The northernmost population of this species, located in the Amur Region, is at risk of flooding by the Lower Bureya Dam. A series of measures were developed and implemented to conserve this plant. Plant individuals were translocated from the flood zone to other suitable habitats on the territory of the Amur Region. Monitoring has indicated that, after three years, the translocated plants have their usual seasonal rhythm, sporulation and the formation of gemmae. The survival rate at new places ranged from 60% to 88%. However, the final conclusions about the research efficiency can be made only after long-term monitoring for more than ten years. Cultivation of this species is being done in outdoor conditions at the Amur Branch of the Far Eastern Botanical Garden-Institute of RAS. The seasonal rhythm of cultivated plants was similar to the natural one in both spore production and gemma formation. The species can be assigned to cold hardiness zone 3 and it is promising for cultivation in the Amur Region. The possibility of cryopreservation of *P. craspedosorum* spores has been studied along with *in vitro* culture. After ultra-low temperatures, spores did not lose their viability and, thus, they can be stored in cryobanks for a long time. An integrated approach provides the maximum opportunity for the species conservation in nature and in culture. This integrated approach to conservation of a rare fern species is unique for Russia.

Key words: cryopreservation of spores, cultivation of ferns, plant conservation, rare species, translocation of ferns

Introduction

At the present time, change in the vegetation cover of our planet, associated with human economic activity, has reached a planetary scale. The consequences of anthropogenic impacts are irreversible changes in natural habitats, and today, in this regard, there is a threat of extinction of both species and ecosystems. Active measures must be undertaken to restore particular plant species and populations in order to minimise the ongoing species loss (Heywood, 2019). The need for research regarding design of methods for practical plant conservation, in particular combining both *in situ* and *ex situ* conservation, is emphasised in the Global Strategy for conservation of plants 2011–2020 (CBD, 2012). Various methods of species conservation do not exclude, but instead complement, one another and should be recognised as integrated programs for restoration of native species *in situ* (e.g. translocation, reintroduction) and *ex situ* (conservation in seed banks and in botanical garden collections) (Ibars & Estrelles, 2012; Ren et al., 2019), keeping a balance between them (Silcock et al., 2019). Various research groups should actively collaborate in a lively exchange of data and, as a corollary, ensure that the necessary level of biodiversity is maintained (Sharpe, 2019).

Most fern species are extremely sensitive to environmental degradation and anthropogenic impact, and are the first to «fall out» of an ecosystem when it is disrupted, which provides the basis for conserving ferns both *in situ* and *ex situ* (Pennisi, 2010; Ballesteros, 2011). Research on questions concerning conservation and restoration of ferns in nature is pressing forward in many countries (e.g. Butz, 2004; Aguraiuja, 2011; Baker et al., 2014; Houser et al., 2016).

In the Amur Region (Far East of Russia), during the construction and filling of the Bureya Dam reservoirs, unique biotic communities and landscapes were practically lost, along with rare and endemic species, such as *Woodsia manchuriensis* Hook., *Cortusa matthioli* var. *sachalinensis* (Losinsk.) T.Yamaz., and *Scrophularia amgunensis* F.Schmidt. The narrowly local endemics *Saxifraga korshinskyi* Kom. and *Taraxacum lineare* Vorosch. & Schaga were reduced to endangered status. The filling of the Lower Bureya Dam (LBD) reservoir and the commissioning of the hydro-unit took place in August 2017, which created the risk of losing unique plant communities and habitats of many relict and rare plant species in the River Bureya valley in the Amur Region. The River Bureya valley is a unique natural refugium of East-Asian flora (Starchenko et al., 2015). At this location, the northernmost habitat of the rare, relict species *Polystichum craspedosorum* (Maxim.) Diels was found. This species has been included in the Red Data Book of the Amur Region (Senchik & Malikova, 2019). The population of this species, discovered in 2011, was small and, according to the predicted water level in the reservoir, was expected to be almost completely drowned. In this connection, the need arose for urgent solutions and actions to conserve the gene pool and the population of *P. craspedosorum*.

The general aim of our study was to design and bring to fruition methods for conserving *Polystichum craspedosorum* both *in situ* and *ex situ*. Our specific objectives were: 1) translocation of plants from the flood zone to suitable habitats in the Amur Region's environment and in culture; 2) studying of the opportunity of long-term storage of *P. craspedosorum* spores by cryopreservation.

Material and Methods

Object of the study

Polystichum craspedosorum is one of the eight representatives of the genus *Polystichum* found in Russia. It is an East Asian species found on the territory of the Russian Far East at the northern limit of its range. The formation of gemmae (or leaf bud, bud brood) on the elongated ends of the frond is a feature of the species, by which plants are capable to vegetative reproduction. In Russia, the distribution of *P. craspedosorum* is limited to the mainland areas of the Russian Far East (Khabarovsky Krai, Primorsky Krai, Amur Region, and the Jewish Autonomous Region). In Khabarovsky Krai and the Jewish Autonomous Region, *P. craspedosorum* is a protected species and has been added to regional Red Data Books (Schlotgauer & Rubtsova, 2006; Voronov et al., 2008). In the Amur Region, plants of this species grow on damp crevices of cliffs along the shore of the River Bureya valley near Sukhiye (Dry) Channels, in the Bureiskiy District (50.0829° N, 130.0969° E) (Starchenko & Darman, 2012).

Translocation of individuals

Translocation of *Polystichum craspedosorum* plants was performed from the part of the population which, according to predictive data, was threatened by flooding. Those plants growing 2 m or more above the projected water level in the reservoir were left on their place. The translocation of *P. craspedosorum* was performed in several stages from 30.05.2015 to 31.07.2017, during various vegetative periods of the fern. Planting was conducted on predetermined plots with optimal conditions based on our observations of the species' habitat requirements (riverside cliffs with a similar geological composition of rocks; with approximate values of abiotic factors, temperature, humidity, illumination): 1) on Zmeinaya Hill (near Domikan village, Arkharinskiy District, 49.70302° N, 129.86493° E); 2) on the left bank of the River Bureya, downstream from the LBD (Bureiskiy District, 49.46362° N, 129.57291° E); 3) on Palminskaya Hill, on the right bank of the River Selemdzha (near the village of Uglovoe, Mazanovskiy District, 51.56336° N, 129.21782° E). The first two plots selected as suitable habitats are part of a Protected Area (PA), Bureiskiy Natural Park (Fig.). The separation of plants into three locations was determined primarily by the intent to minimise the risk of losing plants. At first, if something happen in one place and the translocated individuals die, they would be preserved in another place. Secondly, this separation was determined by land area limitations for plantings, since it was important to plan for the possibility of growth and development of future populations both through vegetative and sporophyte regeneration.

Using small garden shovels, the ferns, together with a layer of soil, were removed from the rocks where they were growing on. In order to reduce injury to the plants, to the extent possible they were not separated from each other but were moved together with turf in small groups. To minimise the root system damage, they were moved in clumps, which made an exact count of individual plants to be difficult. The plants were placed in cardboard boxes and covered with cloth to protect them from solar rays during transporting, and were immediately moved to the planting sites. The plants were carefully watered during the planting process.

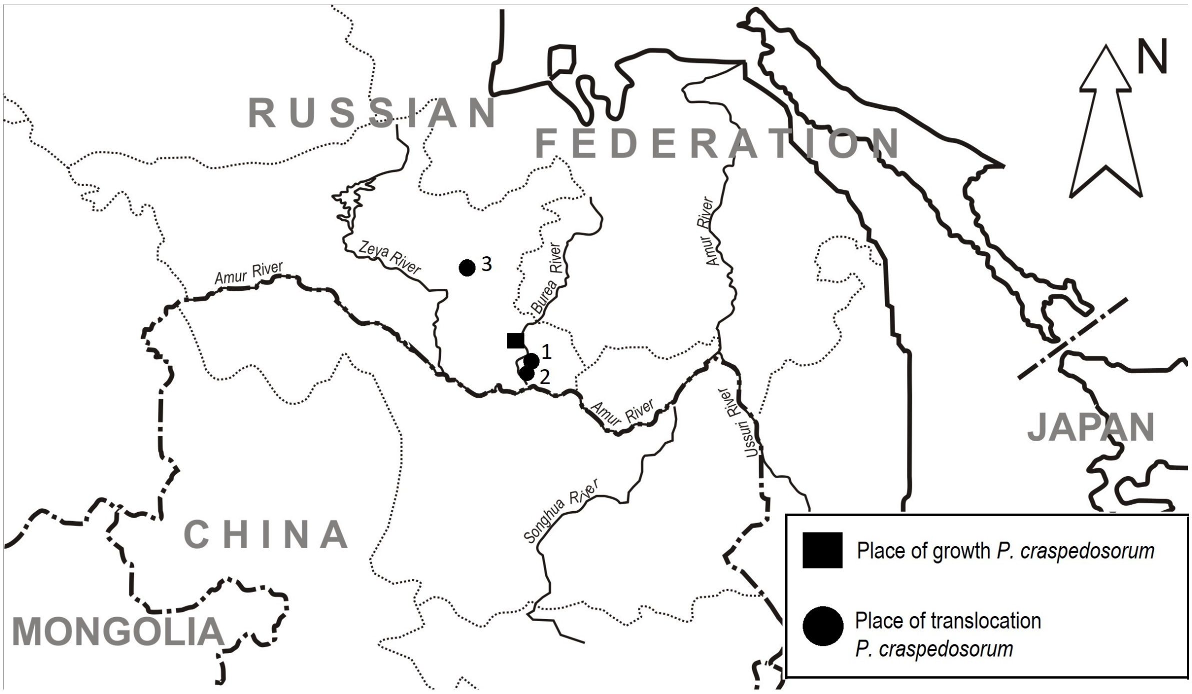

Fig. Locations of growth and translocation of *Polystichum craspedosorum*. Designations: 1 – downstream of the Lower Bureya Dam, 2 – Zmeinaya Hill, 3 – Palminskaya Hill.

Conservation in outdoor fern collections

Outdoor cultivation of *Polystichum craspedosorum* at the Amur Branch of the Far Eastern Botanical Garden-Institute (Blagoveshchensk, Amur Region, Russia, 50.31428° N, 127.4856° E) was initiated at the unique scientific station «Genetic Resources Collection of the Amur Branch, BGI, FEB, RAS». The source of the collected material was represented by 30 living plants from the Sukhiye (Dry) Channels natural habitat, on the right bank of the River Bureya in the Bureiskiy District of Amur Region in 2012, 2016, 2017. Plants were carefully nurtured with regular watering and removal of weeds. Phenological observations were conducted according to Kotukhov (1974). We indicated the beginning of the growing season, the duration of formation and unfolding of fronds, the training of fertile fronds, the beginning of sori formation, the time periods of spore maturation and their pour out, the vegetation termination. Results of the introduction were evaluated according to Khrapko (1989). We noted the winter hardiness, vitality, dimensions and their correlation with those growing in natural surroundings, the occurrence of sporulation, the degree of vegetative growth, the presence of self-sown plants, decorative value, damage by pests and diseases, and others.

Cryopreservation of spores

Polystichum craspedosorum spores were collected for deep freezing from a natural population in the River Bureya valley (Bureiskiy District, Amur Region) in August 2011. Fronds were put into paper packets, which were then placed in shady and well-ventilated spots until spores emerged from sporangia. The germination rate of freshly collected spores was checked. One part of the freshly collected spores was left in paper packets and remained in the laboratory (120 days at a temperature 20–22°C). The other part was put into cryoprobes and immersed in liquid nitrogen (-196°C). The exposure time was 120 days. Defrosting took place in the laboratory air at a temperature of 20–22°C. The experiment was carried out in three replications. The spores were sown in Petri dishes with a diameter of 12 cm with distilled water. Germinated and non-germinated spores were counted in five fields of view using an MBS-10 stereoscopic microscope at 60–80 × magnification. The germination rate of freshly collected spores was used as a control sample. The differences between germinated and non-germinated spores in the control and experiment samples were checked by chi-squared tests with 2 × 2 contingency tables and significance level set at $p < 0.05$ (Zaitsev, 1991). The spore viability was evaluated by germination rate. The germination was expressed in percentages as the relationship of the number of germinated spores to the sum of germinated and non-germinated spores. MS Excel and Statistica v. 10.0 (StatSoft Inc., Tulsa, OK, USA) programs were used for statistical analysis of the results.

Results and Discussion

Translocation of individuals

In our case the method of translocation was chosen because it was necessary to remove (save) mature *Polystichum craspedosorum* plants from the flood zone of the LBD reservoir. In total, about 100 plants were transplanted from the flood zone to safe growing areas: about 53 individuals to Zmeinaya Hill, about 25 individuals to the slopes of hills downstream from the LBD, and 25 individuals to Palminskaya Hill. It should be noted that some authors (e.g. Clabby, 2010; Traill et al., 2010) suggested that 5000 individuals is the minimum number needed for any species of animals or plants to create and further develop a population. Other authors (e.g. Flather et al., 2011) claim that this number is different in each individual case. In some cases, it is sufficient to plant 50 or fewer plants to achieve a stable population (e.g. Silcock et al., 2019). In our case, the number of planted individuals was determined by the numbers of existing *P. craspedosorum* populations that had to be moved from the flood zone.

Annual monitoring of the translocated *Polystichum craspedosorum* plants showed that a large number of them «took root» in their new habitats. Toward the end of the vegetative season, on Zmeinaya Hill, seven dead plants were noted over the entire observation period, three downstream from LBD, and ten on Palminskaya Hill (Table 1). Some of the plants have died from mechanical damage caused by animals.

It should be noted that the plants, developed most successfully, were those, which were transplanted with intact root clumps. In these cases, there were virtually no dead plants, evidently because root systems were less damaged. Transplanted plants typically have a seasonal rhythm: sporification and the formation of gemmae are visible. However, rooting of gemmae has not yet been observed. This allows us to make a tentative conclusion regarding the success of translocation. But since the final goal of translocation is the creation of a sustainable plant population, capable of autonomy, development, and reproduction in a new habitat, final conclusions about the results of this study can be made only after long-term monitoring for at least ten years (Albrecht et al., 2011; Silcock et al., 2019).

Conservation in outdoor fern collections

In the literature on plant cultivation, *Polystichum craspedosorum* appears as a cliff-dwelling, shade-tolerant, winter-hardy species. In its freeze-tolerant zone designation (USDA-zones), it is placed in zones 3–4 (Konovalova & Shevyreva, 2004), zone 5 (Hoshizaki & Moran, 2001), or zones 5–7 (Olsen, 2007). Olsen (2007) notes that growing of *P. craspedosorum* is not always feasible, and can be difficult. It is an obligate calciphile species.

Open-air cultivation of *Polystichum craspedosorum* has been underway since 2014 at the Amur Branch of the Far Eastern Botanical Garden-Institute of RAS. In the collection, there are over 20 individuals of this species. The vegetative season, signaled by the unfurling of fronds, starts in early May. The appearance of new fronds took place during the first ten days of June, and the formation of gemmae in late July or in early August. Spore formation and spore release was started in the second half of August. Fully fledged gemmae were formed in late August or in early September. Rooting of individual gemmae was evident. Sexual regeneration from spores has not been observed. The end of the vegetative season occurs with the onset of prolonged cold weather in late September or in early October. Since temperatures during the winter reach -40°C in natural habitats of this species and in its cultivation location (Blagoveshchensk, Amur Region, Russia), *P. craspedosorum* can be designated as the zone 3 for the frost hardiness.

Table 1. Initial evaluation of *Polystichum craspedosorum* translocation results during 2016–2019

Planting location	Number of translocated individuals	Number of dead individuals	Survival rate
Zmeinaya Hill	53	7	87%
Downstream from Lower Bureya Dam	25	3	88%
Palminskaya Hill	25	10	60%

Table 2. Germination rates of *Polystichum craspedosorum* spores under different storage conditions

Treatment type	Spores, number		Criterion, $\chi2$*	Germination rate
	Germinated	Non-germinated		
Control	338	72	–	82.4%
Cryopreservation (-196°C)	409	76	0.57	84.3%
Laboratory storage (22°C)	421	208	30.33	66.9%

Note: * – if $\chi2 \geq 3.841$, it is a statistically significant difference.

Accordingly, these plants have a typical seasonal rhythm. So, the fronds are green over the winter, being in a twisted, dehydrated condition, and unfurl with the onset of stable above-freezing temperatures and with the appearance of a sufficient amount of soil moisture. In cultivation, *Polystichum craspedosorum* forms fronds, where the spores mature, and gemmae develop. Results permit the conclusion that cultivation is successful, with adequate hardiness in cultivated plants. Khrapko (1996) designates *P. craspedosorum* as a species with excellent potential for cultivation under conditions of the Primorsky Krai, and, in the conditions of Amur Region, this species likewise can be cultivated and included to the group of promising species.

Cryopreservation of spores

Freshly collected *Polystichum craspedosorum* spores were subjected to cryoprocessing to ascertain their extreme low-temperature hardiness and the possibility of using cryopreservation for the long-term storage of genetic material. Results of this experiment are shown in Table 2.

In the control group, the germination rate of spores was 82.4%, and 84.3% after cryoprocessing. Data analysis showed that the germination rate of spores in the control group and after cryopreservation was not significantly different, while germination during storage in laboratory conditions was significantly lower. These data are consistent with data of Ballesteros et al. (2006, 2012), and Kreshchenok & Nesterova (2007) who demonstrated that cryopreservation does not adversely impact the spore viability, while a reliable decrease in germination is observed during laboratory storage. It can be concluded that *Polystichum craspedosorum* spores tolerate the impact of extreme low temperatures and preserve their viability. In this regard, cryopreservation is a promising method for the long-term storage of *P. craspedosorum* spores.

Some authors (e.g. Dudani et al., 2013) emphasise the particular significance of studies concerned to microclonal reproduction of ferns for preservation purposes. One more research was conducted in the Amur Branch of the Far Eastern Botanical Garden-Institute of RAS about *Polystichum craspedosorum* preservation by means of *in vitro* culture, where optimal methods were selected for spore sterilisation, nutrient media for germination and further cultivation of gametophytes and sporophytes, and a process has been developed for sporophyte acclimatisation (Shelikhan, 2020). This provides the possibility of considering cultivation of *P. craspedosorum* gametophytes and sporophytes *in vitro* as a method of conservation of this rare species.

Conclusions

For conservation of populations and gene pools of rare ferns in general, and of *Polystichum craspedosorum* in particular, it is the best way to simultaneously apply both *in situ* and *ex situ* methods. An integrated approach ensures the maximum potential for conserving this species in nature and in culture. Apart from natural habitat conditions, conservation of rare fern species is possible using methods of long-term spore storage through cryopreservation, by *in vitro* culture, and in collections of introduced plants. In the natural environment, it is possible to create new populations in suitable habitats. In addition, monitoring of the status of remaining populations in nature is needed, along with protection of the species in Protected Areas.

Information about integrated conservation of fern populations is absent from scientific, popular-scientific, and mass media publications in Russia. In this regard our study can be considered unique in Russia.

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КОМПЛЕКСНЫЙ ПОДХОД К СОХРАНЕНИЮ РЕДКИХ ВИДОВ ПАПОРОТНИКОВ НА ПРИМЕРЕ *POLYSTICHUM CRASPEDOSORUM* (DRYOPTERIDACEAE) В АМУРСКОЙ ОБЛАСТИ (ДАЛЬНИЙ ВОСТОК РОССИИ)

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Глобальное изменение растительного покрова Земли приобрело планетарный характер. Все значительнее становится опасность исчезновения отдельных видов и экосистем. Необходимо применять активные меры по восстановлению конкретных видов растений и популяций, чтобы минимизировать продолжающуюся потерю видов. *Polystichum craspedosorum* – скальный реликтовый папоротник. Это восточноазиатский вид, находящийся в северной части своего ареала на территории Дальнего Востока России. Самая северная популяция этого вида, находящаяся в Амурской области, попала под угрозу затопления водохранилищем Нижне-Бурейской ГЭС. Для сохранения этих растений был разработан и осуществлен комплекс мероприятий. Была проведена транслокация растений из зоны затопления в другие, подходящие места произрастания, находящиеся на территории Амурской области. Мониторинг показал, что через три года перенесенные растения имеют обычный для себя сезонный ритм, отмечается спороношение, образование выводковых почек. Доля выживания в новых местах составила от 60% до 88%. Однако окончательные выводы о результативности этих работ можно делать только после многолетнего мониторинга, длительностью не менее десяти лет. Осуществляется культивирование этого вида в условиях открытого грунта в коллекции Амурского филиала Ботанического сада-института ДВО РАН. Сезонный ритм культивируемых растений соответствует природному, образуются споры, формируются выводковые почки. Вид отнесен к зоне 3 морозостойкости и является перспективным для культивирования в Амурской области. Исследована возможность криоконсервации спор *P. craspedosorum*, а также введение в культуру *in vitro*. Споры переносят воздействие сверхнизких температур и не теряют свою жизнеспособность. Следовательно, их можно долговременно хранить в криобанках. Комплексный подход обеспечивает максимальную возможность для сохранения вида в природе и культуре. Данный опыт и комплексный подход к сохранению редких видов папоротников является уникальным для России.

Ключевые слова: криоконсервация спор, культивирование папоротников, редкий вид, сохранение растений, транслокация папоротников