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# Phylogeny of the North Asian Cystopteridaceae (Polypodiopsida) based on *trnG-R* intergenic spacer

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The molecular phylogenetic analysis of the Cystopteridaceae family based on sequencing of the plastid DNA intergenic spacer *trnG-R* is carried out. The dataset includes sequences mainly of North Asian samples of both widerspread species and species described from the former USSR and modern Russia. The use of *trnG-R* intergenic spacer showed more comprehensive results for *Gymnocarpium*, than for *Cystopteris*. *Gymnocarpium* phylogeny includes four well-supported clades: the *dryopteris* clade, the *robertianum* clade, the *continentale* clade, and the *jessoense* clade. Data from *trnG-R* support the divergence between species with glabrous fronds (*G. dryopteris*) and species with glandular-pubescent fronds. Among species having glandular-pubescent fronds, the *robertianum* clade is a sister to the remaining ones. *Gymnocarpium fedtschenkoanum* is more related to *G. robertianum*, than other glandular species. Our data confirmed the recognition of *Gymnocarpium continentale* and *G. jessoense* as distinct species. The topology of *G. tenuipes* has remained uncertain. Among *Cystopteris* species three highly supported clades are indicated: the *montana* clade, the *sudetica* clade and the *Cystopteris fragilis* complex. *C. montana* and *C. sudetica* occupy the clearest position in the phylogenetic tree. The topology of *C. montana* as sister to the remainder of the genus, and then the placement of *C. sudetica* as sister to the *C. fragilis* complex does not allow *C. montana* to be included together with *C. sudetica* in the same supraspecific taxon but allows to accept the genus *Rhizomatopteris* as distinct monotypic genus containing a single species – *Rh. montana*. Relationships among the taxa of *C. fragilis* complex including the species described from North Asia remain uncertain.

**Key words**: *Cystopteris*; *Gymnocarpium*; Cystopteridaceae; Plastid DNA; *trnG-R* intergenic spacer; Phylogeny

## Introduction

*Cystopteris* Bernh. and *Gymnocarpium* Newman are both the members of the fern family Cystopteridaceae (Payer) Shmakov, which was first treated as a separate family by A. I. Shmakov (2001), who included in it about 60 species. The family status was accepted and circumscription by M. J. M. Christenhusz et al. (2011), and they recognize 30 species in the family. Subsequent molecular phylogenetic studies provided by C. Rothfels et al. (2012, 2013, 2014) have yielded a clear understanding of this family, which led to the recircumscription of the Cystopteridaceae. In the modern sense, Cystopteridaceae includes of four genera and about 38 species: *Acystopteris* Nakai – 3 species, *Cystoathyrium* Ching – 1 species, *Gymnocarpium* (including *Currania* Copel.) – 7 species, and *Cystopteris* (including *Rhizomatopteris* A. P. Khokhr.) – about 27 species (Rothfels et al., 2013).

Thus, the genera *Cystopteris* and *Gymnocarpium* are the largest genera in this family. Many taxonomists have expressed the complexity of the intrageneric taxonomy of *Cystopteris* and *Gymnocarpium*. As noted by V. N. Siplivinsky (1973: 353), "Difficulties in recognizing of Siberian *Gymnocarpium* are inversely related to their species diversity". Opinion regarding *Cystopteris* is that the *Cystopteris fragilis* complex represents "perhaps the most formidable biosystematics problem in the ferns" (Lovis, 1978: 356).

The molecular phylogenetic study of Cystopteridaceae by C. Rothfels et al. (2013) based on a three-locus plastid dataset (*matK, rbcL, trnG-R*) included multiple accessions of the most species from across their geographic ranges. Authors of that study have established, that all represented genera are supported as monophyletic, the *Gymnocarpium* is deeply divided into three major clades labeled the *disjunctum* clade, the *robertianum* clade, and core *Gymnocarpium*. *Cystopteris* features four deeply diverged clades: *C. montana*, the *sudetica* clade, the *bulbifera* clade, and the *C. fragilis* complex. Only two accessions in the dataset by C. Rothfels et al. (2013) originate from Russia. These accessions represent two species – *Cystopteris sudetica* A. Braun et Milde and *Gymnocarpium jessoense* (Koidz.) Koidz. subsp. *parvulum* Sarvela. C. Rothfels et al. (2013) did not involve several species of Cystopteridaceae described from the territory of the former USSR as well as accessions of widespread species from Russia in their study. Six species of Cystopteridaceae were described from the territory of the former USSR; these are *Gymnocarpium continentale* (Petrov) Pojark., *G. fedtschenkoanum* Pojark., *G. tenuipes* Pojark. ex Shmakov, *Cystopteris almaatensis* Kotukh., *C. altajensis* Gureeva, and *C. gureevae* Stepanov. V. A. Petrov (1930) described *Gymnocarpium continentale* from Yakutia (Russia) as *Dryopteris pulchella* (Salisb.) Hayek var. *continentalis* Petrov, and later A. V. Pojarkova (1950) included it in the genus *Gymnocarpium.* A. V. Pojarkova (1950) described *Gymnocarpium fedtschenkoanum* and *G. tenuipes* from different parts of Central Asia. Protolog of *G. fedtschenkoanum* was published by A. V. Pojarkova (1950) in Latin in compliance with the rules of the botanical nomenclature, the description of *G. tenuipes* was represented in the same publication only in Russian. A. I. Shmakov validated it in 1995 (Shmakov, 1995). *Gymnocarpium fedtschenkoanum* was described from the Hissar Ridge, Tajikistan and considered as endemic for Central Asia (Pamir-Alay, Tianshan, northwest Himalayas). *Gymnocarpium tenuipes* mentioned by A. V. Pojarkova (1950) as an endemic of the Kazakh Uplands, Central Kazakhstan. Afterwards, A. I. Shmakov (1995) indicated it for the Russian Altai. *Cystopteris almaatensis* was described from Zailiisky Alatau, Kazakhstan (Kotukhov, 1966). Latter, this species were found in the Russian Altai (Gureyeva et al., 2015), furthermore, as the authors know on the herbarium collections from LE, MW, and TK, it

occurs also in the mountains of Northern Kyrgyzstan and Tajik Pamir. *Cystopteris altajensis* was described from the Russian Altai (Gureyeva, 1985); its geographic range covers the Altai-Sayan mountain system. And finally, *Cystopteris gureevae* is described recently from Western Sayan (South Siberia, Russia) and is still known only from locations indicated in protolog (Stepanov, 2015).
The aim of this study is the molecular phylogenetic analysis of plastid DNA locus *trnG-R* of the most species of the Cystopteridaceae occurred in North Asia, including species, described from the territory of the former USSR.

## Materials and Methods

Phylogenetic analysis was provided on the base of the sequencing of the intergenic spacer of plastid DNA *trnG-R,* which was used in the study by C. Rothfels et al. (2013). The most of the fern samples were collected in natural populations mainly in South Siberia on the Western Sayan (2015, I. I. Gureyeva, A. A. Kuznetsov, N. V. Stepanov) and Kuznetsk Alatau (2016, I. I. Gureyeva, D. O. Ulko, R. S. Romanets), samples outside Russia were collected by I. I. Gureyeva (Fig. 1). Vouchers are stored in the Herbarium of Tomsk State University (TK). Plastid DNA was extracted from silica-dried material or herbarium specimens using the protocol of the manufacturer of DNA isolation kits or by the standard CTAB method (Rogers & Bendich, 1989). The quality and quantity of DNA were checked on the *Implen P330* spectrophotometer. To obtain comparable results, the amplification of chloroplast DNA locus *trnG-R* was carried out with the same primers used by C. Rothfels et al. (2013) (Table 1 and Figure 1).

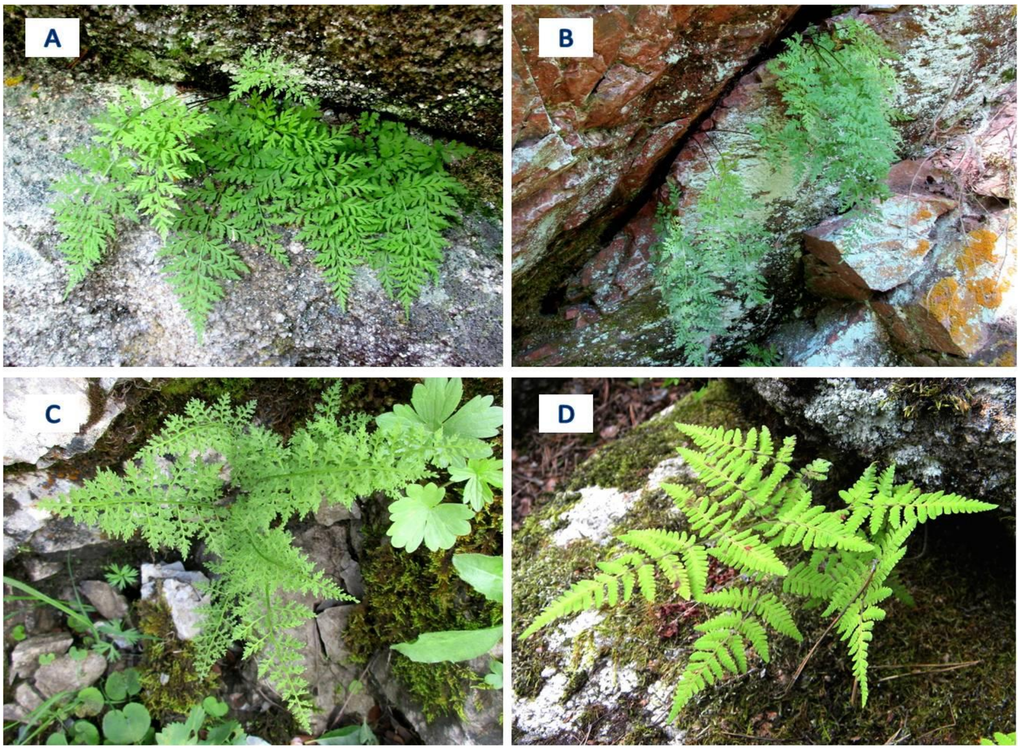

**Figure 1.** Some species of the family Cystopteridaceae. A – *Cystopteris altajensis* (Russia, Kuznetsk Alatau, near Tunguzhul village); B – *Cystopteris gureevae* (Russia, Western Sayan, Malyi Kebezh River); C – *Cystopteris alpina* (Austria, Arlberg, Formarinsee); D – *Gymnocarpium continentale* (Russia, basin of Podkamennaya Tunguska River, mouth of Lakura River). Photos by I. I. Gureyeva (A–C) and Yu. G. Raiskaya (D).

**Table 1.** Primers used for *trnG-R* amplifications of the *Cystopteridaceae* members.

| Locus | Direction | Sequence (5'–3') | References |
|---|---|---|---|
| *trnG-R* | F | GCGGGTATAGTTTAGTGGTAA | Nagalingum et al. (2007) |
| *trnG-R* | F | GCTAYACGACCAARACGTAAGC | Rothfels et al. (2013) |
| *trnG-R* | R | GTGGCATCCATAAAATCYATGTCAG | Rothfels et al. (2013) |
| *trnG-R* | R | CTATCCATTAGACGATGGACG | Nagalingum et al. (2007) |

Loci were amplified in 15 µl reactions consisting of 2 µl of DNA, 1 µl of each primer (10 µM), 1.5 PCR buffer, 1.6 µl of $MgCl^2$, 0.12 µl of dNTP, 0.2 µl of taq polymerase and 7.58 µl of DI water. The PCR reaction was carried out using S1000 Thermal CyclerTM (BIO-RAD) and the thermal cycling program consisted of an initial denaturation step (94ºC for 3 min), 35 denaturation, annealing, and elongation cycles (94ºC for 45 sec, 50ºC for 30 sec, 72ºC for 1.5 min), and a final elongation step (72ºC for 10 min). The obtained amplification was checked for the presence of the target fragment by electrophoresis in 1.5% agarose gel stained with ethidium bromide. After confirming the target fragment for all samples, the purification procedure was carried out: PCR product was treated with a mixture of exonuclease I (to remove dNTP) and shrimp alkaline phosphatase (to inactivate residual primer molecules) at a rate of 1 ea. activity of one and 1 e.a. another activity (15 µl).
The samples treated with the mixture were placed in the thermocycler for 40 min at 37ºC and 20 min at 80ºC. Further, the sequencing reaction was carried out, the mixture of which included: 1 µl of BigDye Sequencing kit v3.1, 1.5 µl of sequencing buffer, 2 µl of one of the primers (forward or reverse), 2 µl of purified PCR product and 3.5 µl of DI water. The amplification program included 25 cycles: 25 sec at 96ºC, 15 sec at 50ºC and 2 min at 60ºC. The resulting product was treated with 2 µl of a mixture of shrimp alkaline phosphatase and DI water at a ratio of 1:1 and placed in the thermocycler for 40 min at 37ºC and 20 min at 80ºC and subsequently precipitated with alcohol and sodium acetate. 2 µl of sodium acetate with blue dextran and 30 µl of chilled 96% ethanol were added to 10 µl of the reaction mixture, whereupon the solution was placed for 10 min at 20°C. After precipitation on

12,000 rpm, the supernatant was collected and 50 µl of 70% ethanol was added to the precipitate, followed by centrifugation for 5 min. After the last supernatant removal, open tubes were dried in an oven at 62ºC for 5 min. The DNA of all samples was prepared for sequencing in the laboratory of structural and molecular analysis of plants at Tomsk State University; sequencing was carried out at Syntol Company (Moscow). All sequences were checked manually. For the phylogram construction, 56 samples collected by authors in the natural populations of 12 species mostly in Siberia were used. In addition, samples collected in some places of Europa (Austria), North America (USA), Central Asia (Tajikistan) were used. The obtained dataset on the sequences of the studied plastid DNA locus was aligned in the Geneiouse program using the MUSCULE algorithm, the phylogram has been constructed using the MEGA X software (Kumar et al., 2018) on the Maximum likelihood (ML) and Mister Bayes (MrB) methods. The consensus tree (phylogram) was constructed as a result of producing of 600,000 generations (25% of the trees are discarded).

# Results

Phylograms based on the dataset of the sequencing of plastid DNA intergenic spacer *trnG-R* with using methods Maximum likelihood (ML) and Mister Byes (MrB) demonstrate the relationship within the Cystopteridaceae and within its constituent genera *Gymnocarpium* and *Cystopteris*, as well as placement of species described from the former USSR territory. In our phylogram, the family includes two strongly supported superclades corresponding to the genera *Gymnocarpium* and *Cystopteris* (including *Rhizomatopteris*) (Figure 2). Superclade of *Cystopteris* genus includes three strongly supported clades, that we following C. Rothfels et al. (2013) called the *montana* clade, the *sudetica* clade, and the *Cystopteris fragilis* complex. The *montana* clade is strongly supported as sister to the remainder of the genus (100% ML bootstrap support/1.0 Bayesian posterior probability); *sudetica* clade is sister to the *Cystopteris fragilis* complex and this topology is also strongly supported (100%/1.0). The feature of the *montana* and *sudetica* clades are their genetic homogeneity. The clade of the *C. fragilis* complex is strongly supported (100%/1.0) and is characterized by a basal dichotomy. One part of the split is quite highly supported (82%/1.0), another part of the split have low support (28%/0.57). Both parts of the dichotomy remain unresolved and are characterized by the unspecific set of species. All accessions of Altai-Sayan *C. altajensis*, accessions *C. dickieana* Sim of transplanted from a *locus classicus* of this species on the Atlantic coast of Scotland and grown in a private garden in Edinburgh, as well as from the Western Sayan (Siberia, Russia), and *C. fragilis* (L.) Bernh. from distant places – Pamir (Tajikistan) and Kuznetsk Alatau (Siberia, Russia) are included in the quite well-supported part. Three low supported part of the *C. fragilis* complex includes *C. fragilis* from different areas (the Altai, the Sayans, the Caucasus, and the Alps), *C. alpina* Desv. from the highlands of Arlberg (the Alps), all accessions of *C. gureevae* and two accessions of *C. dickieana* from the Western Sayan and the Russian Altai, *C. protrusa* (Weath.) Blasdell from Virginia (USA), one accession of *C. altajensis* from Kuznetsk Alatau (Russia), and all accessions of *C. almaatensis* from Pamir (Tajikistan). The highly supported relationship in the *trnG-R* locus is shown by two accessions of species identified as *C. almaatensis* from different locations in Tajikistan (90%/1.0), and two of four accessions of *C. gureevae* (86%/1.0). In general, none of the species of the *C. fragilis* complex included in our study is supported as monophyletic.

In the superclade of *Gymnocarpium*, data from *trnG-R* strongly support four clades: the *dryopteris* clade, the *robertianum* clade, the *continentale* clade, and the *jessoense* clade. The *dryopteris* clade is sister to the remainder of the genus (100% bootstrap support and 1.0 posterior probability). This clade includes *G. dryopteris* (L.) Newman having non-glandular (glabrous) fronds, and taxon with unclear identification (*Gymnocarpium* sp.), which is morphologically similar with *G. dryopteris*, but has scattered glandular pubescence. Another highly supported clade (86%/1.0) contains all accessions of species with glandular-pubescent fronds. Within this clade, the divergence of the *robertianum*-clade with remaining *Gymnocarpium* is strongly supported (100%/1.0). The *robertianum*-clade includes all accessions of *G. robertianum* (Hoffm.) Newman and the single accession of *G. fedtschenkoanum* from Pamir (Tajikistan). The latter merged with *G. robertianum* from the Altai with fairly high support (80%/1.0). The remaining glandular *Gymnocarpium* diverges into the strongly supported subclades (100%/1.0), which we called the *continentale* clade and the *jessoense* clade. The first clade includes all accessions of *G. continentale* and accessions of taxon identified as *G. tenuipes*, the second one contains all accessions of *G. jessoense* (Koidz.) Koidz. The accessions within both subclades demonstrate a great genetic uniformity.

# Discussion

## *Cystopteris* phylogeny

The most significant characteristics of the *Cystopteris* phylogeny based on plastid DNA locus *trnG-R* are the divergence of *C.* (*Rhizomatopteris*) *montana* as the earliest lineage, which is sister to the remainder of the genus, and then the placement of *C. sudetica* as sister to the *C. fragilis* complex (formally subgenus *Cystopteris*). C. Rothfels et al. (2013) have obtained the same topology of *C. montana* and *C. sudetica* in their phylogram based on the combined dataset (*rbcL, matK*, and *trnG-R*). Most of the taxonomists separated *C. montana* and *C. sudetica* from remaining *Cystopteris* based on such morphological features as a long-creeping rhizome and widely-spaced triangular-to-deltoid fronds. R. F. Blasdell (1963) combined these species in the section *Emarginatae* Blasdell. A. P. Khokhryakov (1985) described for these species the distinct genus *Rhizomatopteris* A.P. Khokhr. N. N. Tzvelev (2005), recognizing the Khokhryakov's genus *Rhizomatopteris*, divided it into two sections: the type section *Rhizomatopteris* typified by *C. montana* and the section *Khokhrjakovia* Tzvel. typified by *C. sudetica*. A. I. Shmakov at al. (2018) consider these species to different sections of the subgenus *Emarginatae* (Blasdell) Shmakov of the genus *Cystopteris: C. montana* to the section *Emarginatae*, *C. sudetica* to the section *Khokhrjakovia* (Tzvel.) Shmakov. According to molecular phylogenetic studies conducted by C. Rothfels et al. (2013), topology of *C. montana* in the phylogenetic tree would permit the recognition of the genus *Rhizomatopteris* A. P. Khokhr. However, since A. P. Khokhryakov (1985) included in this genus also *C. sudetica,* "this assemblage is not monophyletic, and there seems little value in recognizing a monotypic *Rhizomatopteris* (containing only *C. montana*)" (Rothfels, 2013: 302). As shown by us earlier (Gureyeva, 2001; Gureyeva, Kuznetsov, 2015; Ulko et al., 2017; Gureyeva et al., 2018), *C. montana* and *C. sudetica* have more differences than similarities in morphological and biomorphological characteristics of sporophytes and ultrastructural characteristics of spores. Their long-creeping rhizomes have a different way of branching and growth: phylogenous branching with monopodial growth in *C. montana* and acrogenic branching with monopodial-dichotomous growth in *C. sudetica*. Fronds are widely spaced and have the different forms: pentagonal with basal basiscopic pinnula on the lowermost pinna longer than adjacent basiscopic pinnula in *C. montana* and deltate with basal basiscopic pinnula on the lowermost pinna shorter than adjacent basiscopic pinnula in *C. sudetica*. Spores in *C. montana* have a perispore with broadly-conical reticulate elements of sculpture, *C. sudetica* has spores with spiny perispore. In general, morphological and molecular features do not allow *C. sudetica* to be include together with *C. montana* in the same supraspecific taxon – a section, subgenus, or genus, since in this case, the supraspecific taxon will be paraphyletic. Based on both molecular and phenetic differences, we consider it is possible to recognize *C. montana* as belonging to the distinct genus *Rhizomatopteris*, especially since the genus was typified by its author A. P. Khokhryakov (1985) by this species.

Plastid locus *trnG-R* has not resolved the *Cystopteris fragilis* complex, including the topology of the species described from Russia and Kazakhstan – *C. altajenses, C. gureevae,* and *C. almaatensis*. *Cystopteris altajensis* and *C. gureevae* are morphologically similar to each other and both differ from *C. fragilis* in form pinnulets (narrow, almost linear, with parallel margins) and presence of scattered, capitate glandular hairs, especially clear visible on the pinnulet margins. Both species produce large spores with length 43–53 (61) µm in *C. altajensis* and 40–61 µm in *C. gureevae* (Gureyeva, Kuznetsov, 2015; Ulko et al., 2017). *Cystopteris altajensis* is hexaploid *n*=126 (Gureyeva et al., 2017). Judging by the similarity in the size of the spores, *C. gureevae* is also hexaploid. Surprisingly, despite the morphological similarity, accessions of *C. altajensis* and *C. gureevae* do not cluster with each other, and mostly enter into different splits of the dichotomy of the *Cystopteris fragilis* complex, although weakly supported (Figure 2). This supports the view that these species are distinct. The single accession of taxon identified as *Cystopteris sp.* from Kuztetsk Alatau (Siberia, Russia) takes an uncertain position in the weakly supported unresolved subclade containing *C. altajensis* from Kuznetsk Alatau, *C. almaatensis* from Pamir (Tajikistan) and *C. protrusa* (USA). *Cystopteris sp.*, which is hexaploid (*n*=126, according to our data) differs from *C. fragilis* by unusual large fronds and by form of pinnae and pinnules. Perhaps it is one of the allopolyploid taxa from the *Cystopteris fragilis* complex. Another problem is the separation of species with rugose spores, traditionally called *C. dickieana*, from species with spiny spores, defined as *C. fragilis*. One of the questions to be resolved is to reveal the relationship between *C. dickieana* from Scotland where this species was described from (Sim, 1848), and mainly the alpine taxon that is characteristic to the mountainous regions of continental Eurasia identified also as *C. dickieana*. Our study was not able to answer this question definitely, although one of the accessions of *C. dickieana* from Siberia (Western Sayan) fell in the same part of the *C. fragilis* complex with the accession *C. dickieana* from Scotland. However, two other Siberian accessions of *C. dickieana* from the Altai and Western Sayan clustered in another part of the *C. fragilis* complex with accessions of another species with rugose spores – *C. almaatensis* from Pamir.

In general, the relationship within *C. fragilis* complex remains uncertain, as well as in the study by C. Rothfels et al. (2013). This would be explained by the wide development of hybridization processes within the complex and reticular evolution via alloploidy that cannot be detected using plastid DNA loci but was proved using the low copy gene *gapCp* of nuclear DNA (Rothfels, 2014).

### ***Gymnocarpium* phylogeny**

The most significant characteristic of the *Gymnocarpium* phylogeny based on plastid DNA locus *trnG-R* is the deep divergence of the genus into four highly supported clades: the *dryopteris* clade, the *robertianum* clade, the *continentale* clade, and the *jessoense* clade. The placement of non-glandular *Gynmocarpium* as sister to the glandular species is similar to results obtained by C. Rothfels et al. (2013) on the base of three plastid loci dataset. Their *disjunctum*-clade includes all accessions of non-glandular species: *G. dryopteris* and *G. disjunctum*; the latter is one of the progenitors of allotetraploid *G. dryopteris* (Pryer, Haufler, 1993; Rothfels et al., 2013, 2014). One of our accessions designated as *Gymnocarpium sp.* is probably a hybrid between *G. dryopteris* and one of the species having glandular-pubescent fronds, most likely *G. continentale*.

Among glangular species of *Gymnocarpium* the *robertianum*-clade is a sister to the *continentale* + *jessoense* clades. Our single accession of *G. fedtschenkoanum* falls in this clade that indicates its greater genetic proximity to *G. robertianum* than to other glandular species. The second feature of this clade is the genetic uniformity of European accessions of *G. robertianum* and highly supported (80%/1.0) association of Altaian *G. robertianum* with Pamirian *G. fedtschenkoanum*. A. V. Pojarkova, an author of *G. fedtschenkoanum*, indicated it as Pamir-Himalayan endemic (Pojarkova, 1950). C. Fraser-Jenkins (2008) also assigns the most of Himalayan *Gymnocarpiium* to *G. fedtschenkoanum.* We expected the placement of *G. fedtschenkoanum* into the *robertianum*-clade, because of the morphological similarity of these species. *G. fedtschenkoanum* distinguishes from *G. robertianum* in the form and dissection of frond lamina, especially in the form of pinnules and pinnulets but is similar in the morphology of the rhizome and spores. The long-creeping rhizome in both species is relatively thick, brown (Gureyeva et al., 2018); their spores have a perispore sculpture consisting mostly of wide, sinuate, anastomosing relatively dense folds, which are perforated or pitted (Gureyeva, Kuznetsov, 2015).

*Gymnocarpium continentale* and *G. jessoense* are very similar in morphological features. Even the complex of different morphological features is not always able to split up these species (Gureyeva et al., 2018). At the same time, these species are both in different strongly supported (100%/1.0) clades, which we named the *continentale* clade and the *jessoense* clade (Figure 2). Despite the fact that the *G. continentale* was described in 1930 (Petrov, 1930) and recognized in 1950 (Pojarkova, 1950), it was widely recognized by Russian taxonomists only since the 80s of the 20th century; at the same years *G. jessoense* was accepted in Russian literature (Gureyeva, 1984; 2001; Shmakov, 1995, 2005, 2009, 2011). Outside of Russia, these taxa are considered as subspecies: *G. jessoense* subsp. *parvulum* and *G. jessoense* subsp. *jessoense. Gymnocarpium jessoense* subsp. *parvulum* was described by J. Sarvela (1978) in return of *G. continentale*, because he did not find the type specimens of Petrov's *Dryopteris pulchella* var. *continentale* in LE (Saint-Petersburg, at that date Leningrad). In 1978, he included *G. continentale* as a synonym in his widespread *G. jessoense* subsp. *parvulum*. Later, J. Sarvela changed his mind about subspecific status of taxa, mainly because the East Asian *G. jessoense* subsp. *jessoense* is diploid with *n*=40 and *G. jessoense* subsp. *parvulum* is tetraploid with *n*=80 (Sarvela et al. 1981). Afterward, *G. jessoense* subsp. *parvulum* was mentioned as *G. continentale* in the work with Sarvela's participation (Pelinen et al., 1999). C. Rothfels et al. (2013) based on the results of molecular phylogenetic study have concluded, the "placement of *G. jessoense* subsp. *jessoense* (diploid) and *G. jessoense* subsp. *parvulum* (tetraploid) in different well-supported clades suggests that these taxa should be treated as distinct species" (Rothfels et al., 2013: 302). In their next phylogenetic study based on the nuclear locus, C. Rothfels et al. (2014) proved that these taxa should be considered as different species; *G. jessoense* subsp. *parvulum* has an allopolyploid origin and the most appropriate name for it is *G. continentale* (Petrov) Pojark. C. Rothfels's investigations (Rothfels et al., 2013) included two accessions of taxon identified as *G. jessoense* subsp. *parvulum* from Canada and from Sakhalin (Russia). Our three accessions originate from the different places of Siberia, from where was described *G. continentale*. These accessions form clade deeply split from clade included all accessions of *G. jessoense*. Thus, this result is the additional evidence of the independence of these two taxa and their species status. All the more so, those type specimens, the absence of which was an obstacle to the recognition of *G. continentale*, were found, the name was typified, and the lectotype was designated by I. I. Gureyeva (2010). Two accessions of *Gymnocarpium tenuipes* fell in the *continentale* clade. As mentioned above, *Gymnocarpium tenuipes* was indicated by A. I. Shmakov (1995) for the Russian Altai, but not for other Siberian territories. Our accessions of taxon identified as *G. tenuipes* collected in the Altai and northwestern part of the Eastern Sayan Mountains (State reserve "Stolby") show the plastid locus *trnG-R* uniformity with accessions of *G. continentale*. This may be evidence that specific identification of the herbarium specimens was wrong and in fact, they belong to *G. continentale*. For the definition of the species status of *G. tenuipes* and its relationship with other *Gymnocarpium* species, the samples from the *locus classicus* of *G. tenuipes* in the Kazakh Upland is required.

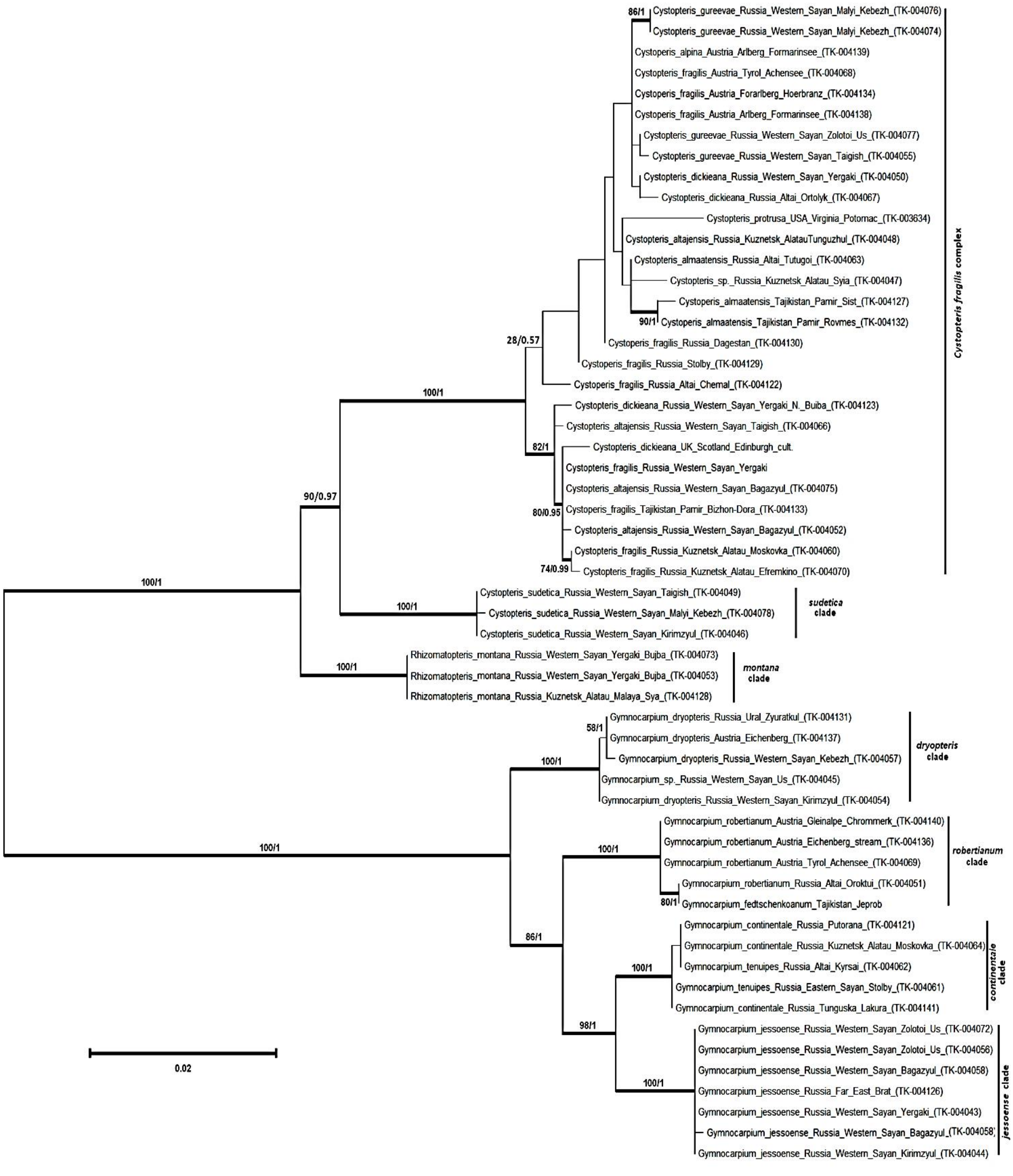

**Figure 2**. Maximum likelihood phylogram based on the sequences of the plastid *trnG-R* intergenic spacer. Support values (maximum likelihood bootstrap support/Bayesian posterior probability) are below the branches. Thickened branches are highly supported (≥70% maximum likelihood bootstrap support and ≥0.95 posterior probability). Numbers in brackets indicate the vouchers stored in the Herbarium of Tomsk State University (TK) (See Appendix).

## Conclusion

The molecular phylogenetic analysis of the North Asian members of the family Cystopteridaceae based on the sequencing of *trnG-R* intergenic spacer of plastid DNA showed more comprehensive results for *Gymnocarpium*, than for *Cystopteris*. *Gymnocarpium* phylogeny includes four well-supported clades: the *dryopteris* clade, the *robertianum* clade, the *continentale* clade, and the *jessoense* clade. Data from *trnG-R* support the divergence between species with glabrous fronds and species with glandular-pubescent fronds. Among glangular species of *Gymnocarpium*, the *robertianum* clade is a sister to other species having glandular-pubescent fronds. *Gymnocarpium fedtschenkoanum* is more related to *G. robertianum*, than other glandular species. Our data confirmed the recognition of *Gymnocarpium continentale* and *G. jessoense* as distinct species. The topology of *G. tenuipes* has remained uncertain. Among *Cystopteris* species, *C. montana* and *C. sudetica* occupy the clearest position in the phylogenetic tree. The topology of *C. montana* as sister to the remainder of the genus, and then the placement of *C. sudetica* as sister to the *C. fragilis* complex does not allow *C. montana* to be included together with *C. sudetica* in the same supraspecific taxon but allows to accept the genus *Rhizomatopteris* as a distinct monotypic genus containing a single species – *Rh. montana*. Relationships among the taxa of *C. fragilis* complex including the species described from the former USSR and modern Russia remain uncertain.

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## Appendix

List of accessions sampled in this study, presented in the following format: Species, Voucher: locality, date of collection, collector's name (Herbarium acronym and number of specimen).

## Studied specimens

***Cystopteris almaatensis***: Russia, the Altai, Tutugoi, 15.07.2000. A. Ebel (TK-004063); Tajikistan, Pamir, Sist, 11.08.2017, M. Olonova (TK-004127); Tajikistan, Pamir, Rovmes 17.08.2017, M. Olonova (TK-004132);

***C. altajensis***: Russia, Western Sayan, Taigish, 15.07.2015, I. Gureyeva et al. (TK-004066); Russia, Western Sayan, Bagazyul, 13.07.2015, I. Gureyeva et al. (TK-004052, TK-004075); Russia, Kuznetsk Alatau,Tunguzhul, 26.06.2016, I. Gureyeva et al. (TK-004048);

***C. dickieana***: Russia, Western Sayan, Yergaki, 17.07.2015, I. Gureyeva et al. (TK-004050); Russia, Western Sayan, N. Buiba, 14.07.2015, I. Gureyeva et al. (TK-004123); Russia, Altai, Ortolyk, 27.07.2014, A. Revushkin et al., (TK-004067); UK, Scotland, Edinburgh, H. McHaffie, cult.;

***C. fragilis***: Austria, Forarlberg, Hoerbranz, 25.08.2019. I. Gureyeva (TK-004134); Austria, Tyrol, Achensee, 11.08.2013. I. Gureyeva (TK-004068); Austria, Arlberg, Formarinsee, 25.08.2019. I. Gureyeva (TK-004138); Tajikistan, Pamir, Bizhon-Dora, 08.08.2017. M. Olonova (TK-004133); Russia, Dagestan, 24.06.2018, I. Gureyeva (TK-004130); Russia, Stolby, 29.07.2016, V. Kurbatskiy (TK-004129); Russia, Altai, Chemal, 03.07.2006, I. Gureyeva (TK-004122); Russia, Kuznetsk Alatau, Efremkino, 08.08.2015, A. Ebel (TK-004070); Russia, Kuznetsk Alatau, Moskovka, 20.07.2015, A. Ebel (TK-004060), Russia, Western Sayan, Yergaki, 14.07.2015, I. Gureyeva et al.;

***C. gureevae***: Russia, Western Sayan, Taigish, 15.07.2015, I. Gureyeva et al. (TK-004055); Russia, Western Sayan, Malyi Kebezh, 16.07.2015, I. Gureyeva et al. (TK-004074; TK-004076); Russia, Western Sayan, Zolotoi Us, 17.07.2015, I. Gureyeva et al. (TK-004077);

***C. alpina***: Austria, Arlberg, Formarinsee, 25.08.2019. I. Gureyeva (TK-004139);

***C. sudetica***: Russia, Western Sayan, Taigish, 15.07.2015, I. Gureyeva et al. (TK-004049); Russia, Western Sayan, Kirimzyul, 13.07.2015, I. Gureyeva et al. (TK-004046); Russia, Western Sayan, Malyi Kebezh, 16.07.2015, I. Gureyeva et al. (TK-004078);

***Cystopteris*** sp.: Russia, Kuznetsk Alatau, Syia, 24.06.2016, I. Gureyeva et al. (TK-004047);

***Rhizomatopteris montana***: Russia, Western Sayan, Yergaki, Bujba, 14.07.2015, I. Gureyeva et al. (TK-004053, TK-004073); Russia, Kuznetsk Alatau, Syia, 25.06.2016, I. Gureyeva et al. (TK-004128);

***Gymnocarpium continentale***: Russia, Putorana, 21.07.2014, I. Volkova et al. (TK-004121); Russia, Tunguska, Lakura, 6.07.2014, Yu. Raiskaya (TK-004141); Russia, Kuznetsk Alatau, Moskovka, 19.07.2015, A. Ebel (TK-004064); ***G. dryopteris***: Russia, Western Sayan, Kebezh 13.07.2015, I. Gureyeva et al. (TK-004057); Russia, Western Sayan, Kirimzyul, 13.07.2015, I. Gureyeva et al. (TK-004054);

***G. fedtschenkoanum***: Tajikistan, Jeprob, M. Nobis;

***G. jessoense***: Russia, Western Sayan, Yergaki, 14.07.2015, I. Gureyeva et al. (TK-004043); Russia, Western Sayan, Kirimzyul, 13.07.2015, I. Gureyeva et al. (TK-004044); Russia, Western Sayan, Zolotoi Us, 17.07.2015, I. Gureyeva et al. (TK-004072, TK-004056); Russia, Western Sayan, Bagazyul, 13.07.2015, I. Gureyeva et al. (TK-004058); Russia, Far East, Brat, 01.09.2013, A. Kuznetsov et al., (TK-004126);

***G. robertianum***: Russia, Altai, Oroktui, 03.0702005, A. Pyak (TK-004051); Austria, Gleinalpe, Chrommerk, 11.07.2009, A. Shmakov et al. (TK-004140); Austria, Eichenberg, stream, 24.08.2019, I. Gureyeva (TK-004136); Austria, Tyrol, Achensee, 11.08.2013, I. Gureyeva (TK-004069);

***G. tenuipes***: Russia, Altai, Kyrsai, 07.2006. I. Gureyeva et al. (TK-004062); Russia, Eastern Sayan, Stolby, 28.07.2016, V. Kurbatskiy (TK-004061);

***Gymnocarpium*** *sp.*: Russia, Western Sayan, Us, 17.07.2015, I. Gureyeva et al. (TK-004045).

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