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Intron length polymorphism of β-tubulin genes in *Colobanthus quitensis* across the Argentine Islands-Kyiv Peninsula region

Abstract. This work analyses intron length polymorphism of β-tubulin genes in populations of Antarctic pearlwort (*Colobanthus quitensis*) from the relatively compact region of the Argentine Islands-Kyiv Peninsula (the maritime Antarctic). Analysis of the length polymorphism of the two introns of β-tubulin genes in natural populations of *C. quitensis* revealed a generally low level of genetic polymorphism. Investigation of the first intron length polymorphism revealed two groups of populations. The population of the largest of Berthelot Islands has representatives of both groups. The second intron length polymorphism of β-tubulin genes identified individual genotypes in 7 of the 11 studied populations of *C. quitensis*. We speculate that this might be due to the spread of plants from different locations or a combination of changes under different environmental conditions.

Keywords: Antarctic pearlwort, Antarctic Peninsula, genetic polymorphism, natural populations

1 Introduction

The genus *Colobanthus* (Caryophyllaceae) is mainly distributed in the Southern Hemisphere and is particularly species-rich in New Zealand (Allen, 1961, cited in Alban et al., 2022). This is confirmed by recent data that the ancestor of *Colobanthus* was distributed in the Southern Hemisphere, in "Australasia" or "New Zealand" (Alban et al., 2022). *Colobanthus quitensis* (Kunth) Bartl. (1831), also known as the Antarctic pearlwort, occurs in South and Central America (with its northernmost location in the mountainous part of Mexico), South Georgia and other sub-Antarctic islands,

several islands of the Southern Indian Ocean (e.g., Kerguelen Islands), Australia (including Tasmania) and New Zealand. At the same time, this species is not found in Africa and New Guinea (Alban et al., 2022). The distribution path of *C. quitensis* remains unclear. According to Alban et al. (2022), two different directions of spreading are possible: from "Australasia" or "New Zealand" to South America *via* the Sub-Antarctic and the opposite. Biersma et al. (2020) proved that maritime Antarctic populations consisted of two different haplotype clusters (based on analysing one nuclear and four plastid markers) occupying the northern and the southern maritime Antarctic. The elucidation of the distribution directions of *C. quitensis* is complicated by the fact that studies have focused on large regions without considering the development of its populations in each particular area of the Antarctic. Previous analysis of individual genetic markers does not yet allow us to draw unequivocal conclusions regarding the evolution of *C. quitensis* in the Antarctic region (Lee & Postle, 1975; Androsiuk et al., 2015; Koc et al., 2018). For that reason, the search for new markers that could be used for biogeographical analysis in maritime Antarctica remains an urgent issue. For instance, the combined analysis of DNA markers (Biersma et al., 2020) showed that populations from maritime Antarctica are divided into two distinct groups: one sampled from the southern part of the Antarctic Peninsula, and the other sampled from the South Shetland Islands and the northern part of the Antarctic Peninsula. However, the distribution routes of *C. quitensis*, both in the Southern Hemisphere in general and in maritime Antarctica in particular, are still under question. This is mainly due to logistical and technical constraints, such as limited geographical sampling and small sample sizes, as well as the unsuitability of the markers used for molecular dating techniques.

Antarctic pearlwort was studied using a number of molecular genetic markers. In previous studies, isoenzyme analysis (Lee & Postle, 1975) and ITS rDNA sequences (Gianoli et al., 2004; Acuna-Rodríguez et al., 2014; Biersma et al., 2020; Alban et al., 2022) were used to establish the Antarctic pearlwort's genetic characteristics. Over the past years, its chloroplast genome sequence has been determined (Kang et al., 2016), and its genome size has been estimated (Cuba-Diaz et al., 2017; Pascual-Diaz et al., 2020). Genetic polymorphism between the southern and northern groups of populations in Antarctica was assessed using retrotransposon-based DNA markers (iPBS) (Androsiuk et al., 2015; Koc et al., 2018).

In recent years, markers based on the assessment of intron length polymorphism (ILP) have gained considerable popularity in population genetic studies (Gowd et al., 2023). They have been shown to be a fast, simple, reliable, and versatile method of plant genotyping that does not require significant prior information about genomes and allows different taxonomic units to be differentiated from each other. Their high efficiency has been demonstrated by a number of recent studies (Braglia et al., 2021; Lykholat et al., 2022). One of the most successful ILP markers is based on assessing intron length polymorphism in the β-tubulin gene family of different plant species (Tubulin-Based-Polymorphism, TBP). TBP is a method widely applicable to any plant species and is particularly suitable for the first and rapid classification of any plant genome (Braglia et al., 2023). In some cases, a modification of the TBP method is used – combinatorial TBP or cTBP (Braglia et al., 2010). It is based on the analysis of the length of the second intron of the β-tubulin gene. cTBP is very useful when plants cannot be differentiated by the first intron of the β-tubulin genes. A low level of genetic polymorphism in natural populations of *C. quitensis* was shown using four DNA markers based on intron length polymorphism of cytoskeletal protein genes (actin, α- and γ-tubulin) (Rabokon et al., 2020). Besides, the TBP method proved to be an effective system for identifying different ecotypes of Antarctic vascular plants (Rabokon et al., 2018), which can therefore be used to study the genetic differences between different populations of *C. quitensis*.

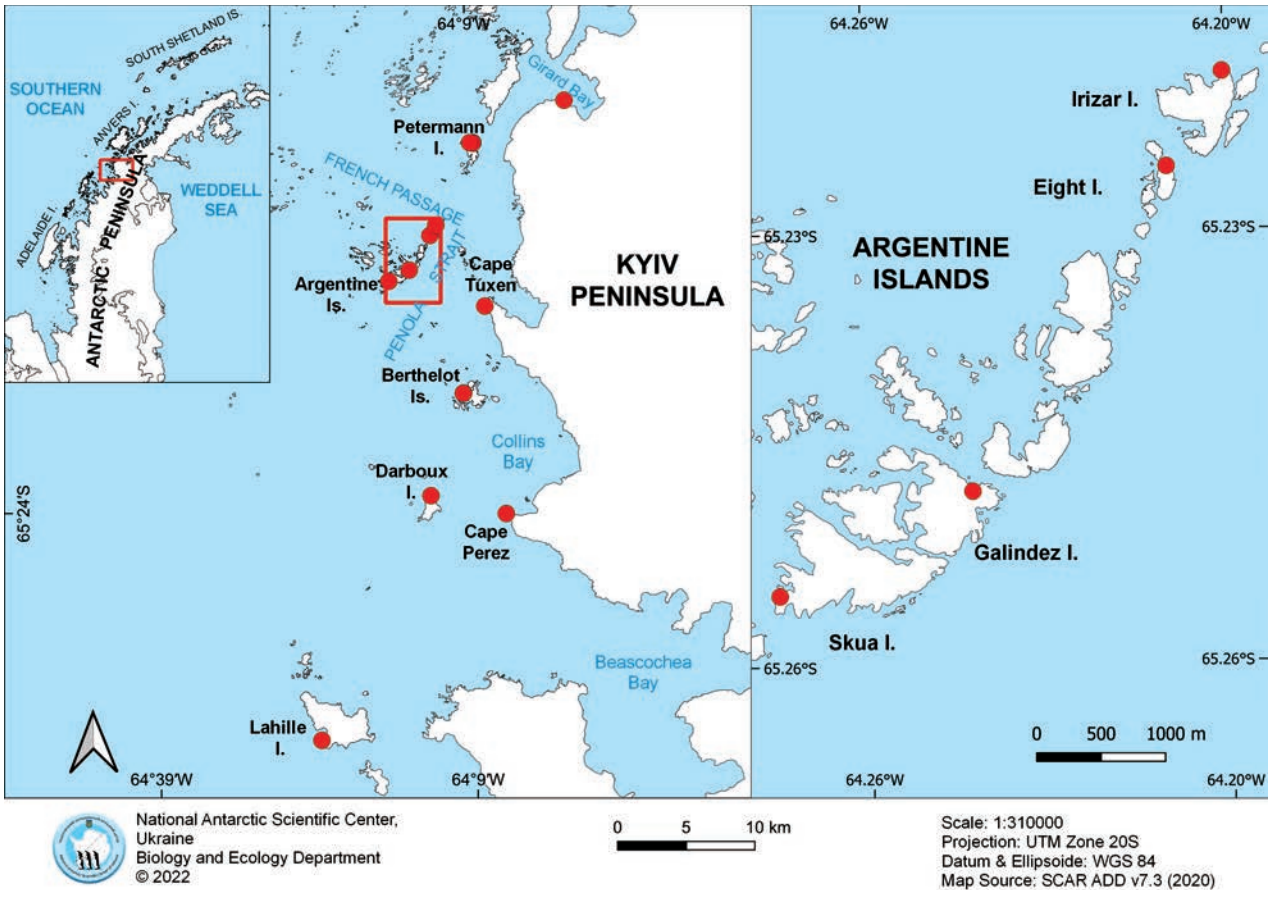

Figure 1. Map of the sampled populations of *Colobanthus quitensis* in the Argentine Islands-Kyiv Peninsula region

In view of the above, we aimed to assess the interpopulation heterogeneity of *C. quitensis* in the Argentine Island-Kyiv Peninsula region using new effective molecular markers.

2 Materials and methods

2.1 Population sampling

The geographical locations of the sampled populations are shown in Figure 1, and the coordinates of sampling sites are presented in Table 1. Please see Yevchun et al. (2021) for information about the region's topography. Samples were collected during the 24th (2019/2020) and the 25th (2020/2021) Ukrainian Antarctic expeditions. They include both those described earlier (Smith & Corner, 1973) as well as those found in the region in 2014–2021. The material was instantly packed into paper bags and stored with silica gel for further use.

2.2 Tubulin-based polymorphism detection

Genomic DNA was extracted with cetyltrimethylammonium bromide (CTAB method) (Murray & Thompson, 1980). The QIAGEN DNeasy Plant Mini Kit was used per the manufacturer's protocol. Then, the quality and quantity of DNA were assessed by electrophoresis in 1.5% agarose gel. The degree of purity and DNA concentration were tested spectrophotometrically using Nanodrop One. The DNA samples were stored at −20 °C.

TBP and cTBP analyses were performed according to Breviario et al. (2007). The sequences of all primers used for the polymerase chain re-

action (PCR) are shown in Table 2. PCR was performed on the ThermalCycler 2720 amplifier (Applied Biosystems, USA). The reaction mixture (25 µl) contained five-fold PCR buffer with ammonium sulfate, 2.5 µM $MgCl_2$, 50 ng plant DNA, 1 µM each primer, 0.2 µM each dNTP, 0.5 units Taq polymerase (Thermo Scientific, USA).

The amplifications were performed according to the following protocol: initial denaturation (94 °C) – 3 min, 38 cycles of amplification (denaturation 94 °C – 30 s, primer annealing 55 °C – 40 s, elongation 72 °C – 1.5 min), final elongation 72 °C – 8 min, 15 °C – retention.

The amplification products were separated by electrophoresis in 6% non-denaturing polyacrylamide gel in 1 × TBE buffer at 380 V. DNA fragments were visualized by silver nitrate staining. After the electrophoretic analysis, the gel was photographed in visible light and the obtained images were analysed with the GelAnalyzer software program (http://www.gelanalyzer.com). The length of reproducible and clear DNA fragments was determined using a DNA marker (O'Gene Ruler™ 100bp Plus DNA Ladder, ready-to-use; Thermo Scientific, USA).

Each PCR reaction was performed at least twice with negative control and was followed by electrophoretic analysis to detect the nonspecific amplification products that differed between the same reactions. If the results of PCR coincided, we took into account all the bands, but if the results were different, we carried out the second amplification and took into account only the bands that were common for all PCR reactions. In all cases,

Table 1. Locations of the sampled *Colobanthus quitensis* populations from the Argentine Islands-Kyiv Peninsula region

Location	Geographical coordinates	Number of samples
The largest of the Berthelot Islands (Ukraine Island)	−65.329040°, −64.161650°	10
Eight Island (largest island SW from Irizar Island)	−65.225630°, −64.209630°	10
Cape Tuxen	−65.272420°, −64.126000°	6
Galindez Island	−65.247967°, −64.242600°	8
Cape Pérez	−65.407530°, −64.097290°	7
Northern part of Petermann Island, loci 1–3	−65.165530°, −64.140150°; −65.165489°, −64.144440°; −65.165710°, −64.152150°	12
Lahille Island	−65.553605°, −64.395064°	9
Girard Bay	−65.138494°, −64.001890°	9
Skua Island, Finger Point	−65.255050°, −64.275040°	10
Darboux Island	−65.395246°, −64.215358°	7
Irizar Island	−65.218970°, −64.200050°	6

Table 2. The primers used for the sampled *Colobanthus quitensis* populations from the Argentine Islands-Kyiv Peninsula region

Markers	Primers (5'-3')	
	F	R
TBP (1st intron of β-tubulin) cTBP (2nd intron of β-tubulin)	AACTGGGCBAARGGNCAYTAYAC GARAAYGCHGAYGARTGYATG	ACCATRCAYTCRTCDGCRTTYTC CRAAVCCBACCATGAARAARTG

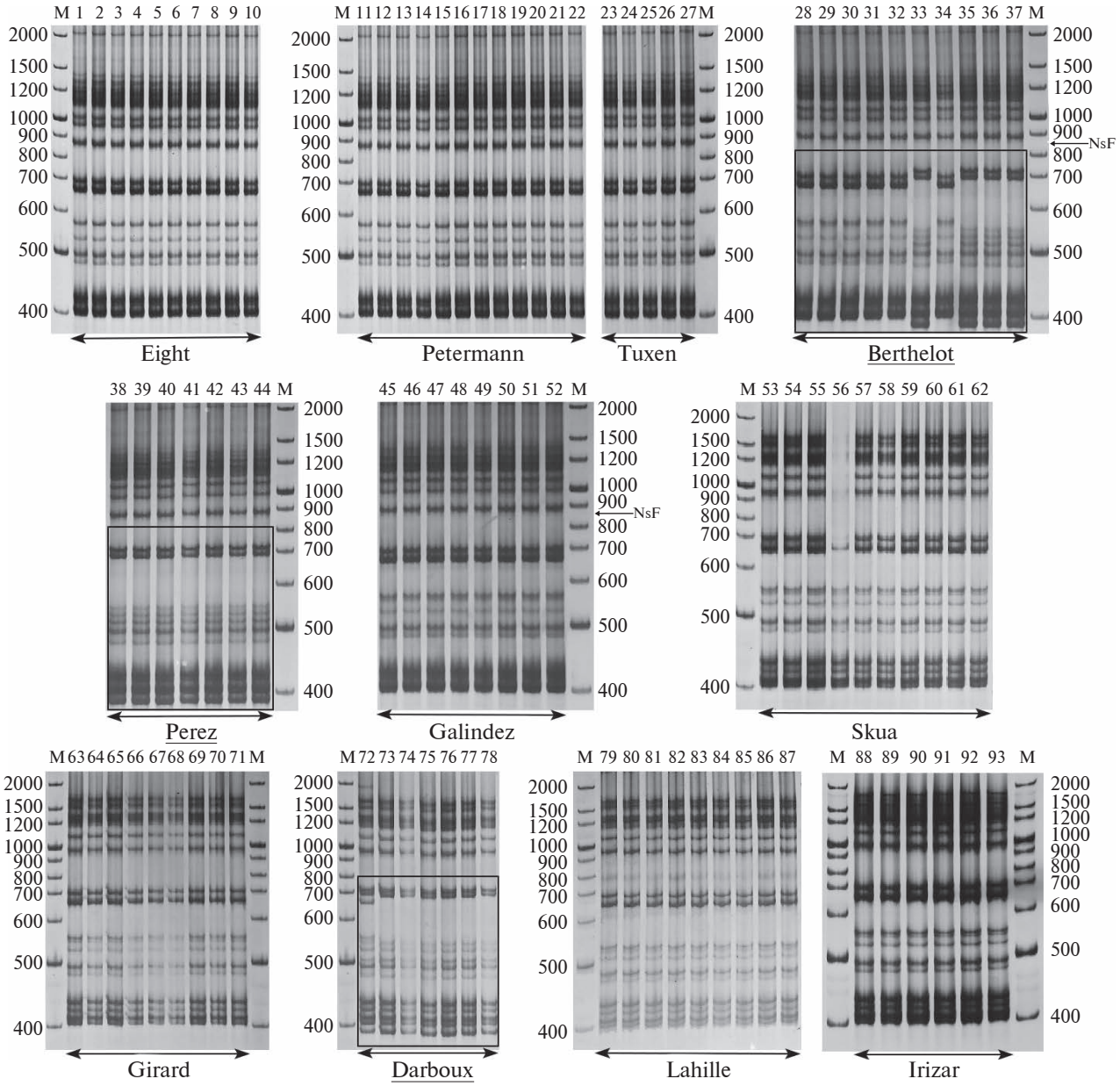

Figure 2. Electrophoretic spectra of the amplified fragments containing the first intron of β-tubulin genes for the sampled *Colobanthus quitensis* populations from Argentine Islands-Kyiv Peninsula region: 1–93 (above) – numbers of population samples; M – DNA marker 100bp Ladder; NSF – nonspecific DNA fragment. Rectangles indicate areas of polymorphic fragments

we scored only reproducible and clear bands. Bands were scored as a binary system: present = 1, absent = 0. Genetic distances between pairs of genotypes were determined by the Free Tree program (Pavlicek et al., 1999) from the matrix of presence/absence of amplified bands of the analysed samples, according to (Nei & Li, 1979). The similarity values were used for the cluster analyses and building of phylogenetic trees, plotted as dendrograms according to the unweighted pair group

method with arithmetic average (UPGMA (Sneath & Sokal, 1973)). Clustering results were scored based on 1000 bootstraps (Hillis & Bull, 1993) by the same program. The resulting dendrograms with bootstrap support were visualized by the Itol v6 program (https://itol.embl.de/).

3 Results

93 DNA samples from 11 isolated *C. quitensis* populations of the Argentine Island-Kyiv Peninsula region were examined using the TBP method, based on estimating the first intron length polymorphism of β-tubulin genes. The electrophoretic analysis of all samples allowed identifying the formation of DNA fragments in the range from 380 bp to 1900 bp (Fig. 2). Fragments up to 1200 bp were probably the targets, as they were the most distinct among all runs of the experiment. These fragments were further analysed. Longer fragments were discarded as artifacts. A DNA fragment of approximately 880 bp was also excluded from the analysis as nonspecific, as it was detected only in a subset of the experiments.

All samples from eight populations (Eight Island, Cape Tuxen, Galindez Island, the northern part of Petermann Island, Lahille Island, Girard Bay, Finger Point of Skua Island, Irizar Island) and 6 of 10 samples from the largest of Berthelot Islands had an identical DNA profile, consisting of 12 amplicons from 400 bp up to 1200 bp in length. Concerning the samples from Darboux Island and Cape Pérez, as well as 4 of the 10 samples from the largest of Berthelot Islands, their DNA profiles, albeit containing most of the fragments in common with other samples, had additional specific fragments. These were: 380 bp, 465 bp, 510 bp, 540 bp, and 725 bp. In these samples, there were no DNA fragments with a length of 475 bp, 565 bp, and 675 bp detected in the samples from other populations. The only exception was sample 72 (Darboux Island), which, apart from additional unique fragments, also contained 565 bp and 675 bp fragments.

In terms of DNA polymorphism, the populations of Darboux Island and Cape Pérez were the most different. At the same time, the population of the largest of Berthelot Islands was in-between the two groups of populations: the Eight, Galindez, northern part of Petermann, Lahille, Skua, Irizar Islands, Cape Tuxen, and Girard Bay as group one, which showed very similar patterns, and the Darboux Island and Cape Pérez as group two, which were somewhat different from the other ones. For this reason, it was concluded that the population of the largest of Berthelot Islands combined some features of the genotypes of the two groups.

Overall, the results of the first intron length polymorphism of the β-tubulin genes indicate that among the studied Antarctic pearlwort populations, those from Darboux Island, Cape Pérez, and some of the population samples from the largest of Berthelot Islands stand out. This may indicate different distribution scenarios and microevolutionary processes dependent on local abiotic and biotic factors.

For further analysis, the cTBP method was used to assess the polymorphism of the second intron length of the β-tubulin genes. Sixteen monomorphic amplicons ranging from 300 bp to 1200 bp were formed in all samples (Fig. 3).

Some samples stood out as they had additional fragments in their DNA profiles. In particular, samples 16, 20, 21 (northern part of Petermann Island), 49 (Galindez Island) and 82 (Lahille Island) had a 625 bp fragment; samples 5 (Eight Island), 23 (Cape Tuxen) and 33 (the largest of Berthelot Islands) contained 400 bp and 410 bp fragments; samples 8 (Eight Island), 29 (the largest of Berthelot Islands) and 91 (Irizar Island) had one fragment 400 bp long; and sample 7 (Eight Island) possessed a 410 bp fragment.

Thus, by analysing the second intron length polymorphism of β-tubulin genes, individual genotypes were identified in 7 out of the 11 studied *C. quitensis* populations. These were the largest of the Berthelot Islands, Eight, Galindez, the northern part of Petermann, Lahille, Irizar islands, and Cape Tuxen. All the remaining populations: Girard Bay, Skua Island, Cape Pérez, and Darboux

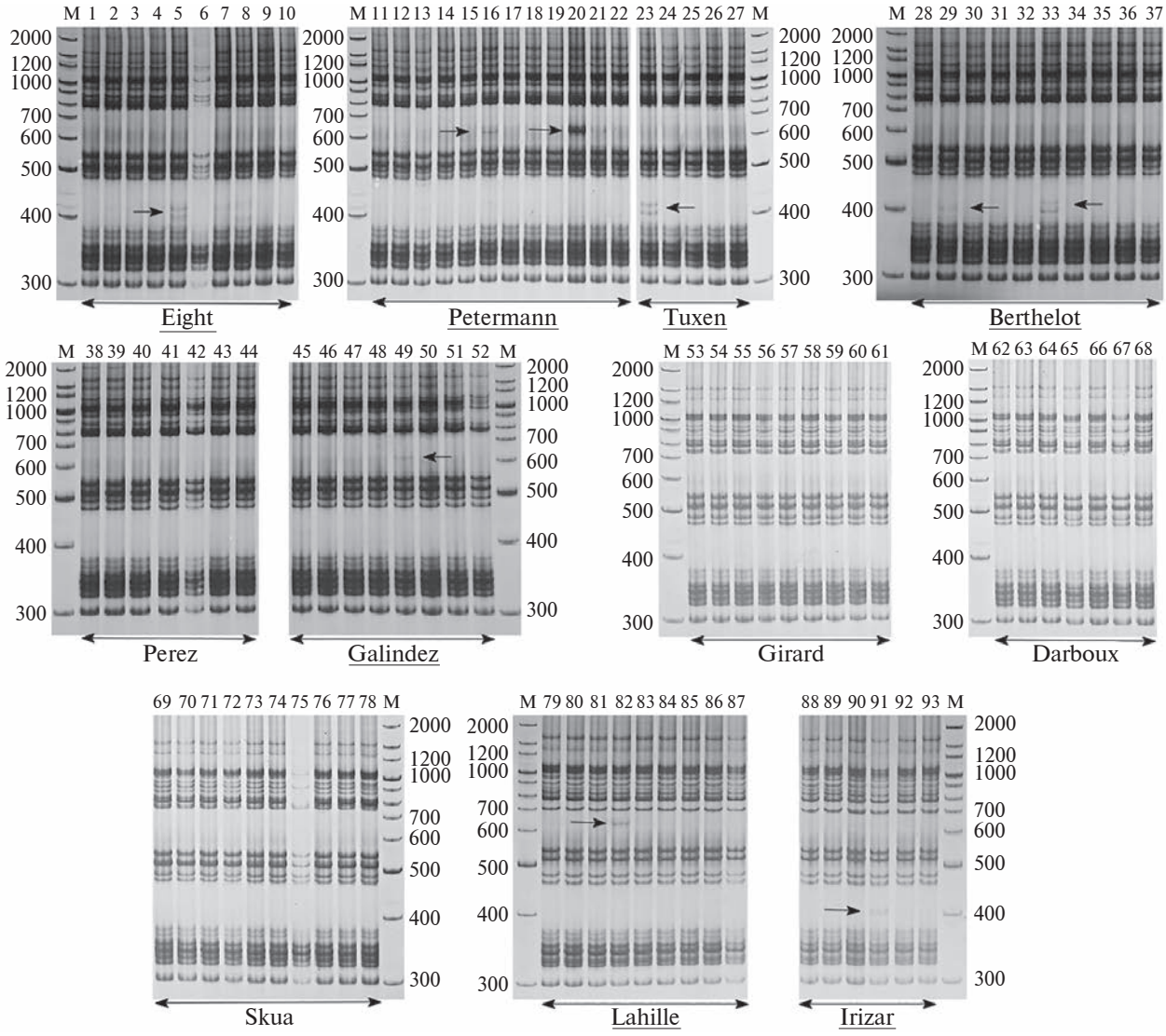

Figure 3. Electrophoretic spectra of amplified fragments containing the second intron of β-tubulin genes for the sampled *Colobanthus quitensis* populations from the Argentine Islands-Kyiv Peninsula region: 1–93 – numbers for sampled populations. M – DNA marker 100bp Ladder. Arrows indicate areas of polymorphic fragments

Island, had the same genetic variants without individual traits according to the studied markers.

4 Discussion

Until lately, it was generally believed that modern Antarctic biota came to exist relatively recently. The present-day climate change challenge has left many indigenous species with little chance of survival (Convey et al., 2008). However, a number of studies have shown that a significant number of species from major groups of living organisms (bacteria, invertebrates, lichens, mosses, etc.) did not completely disappear during the glaciation but only underwent significant reductions in numbers (Convey et al., 2008; Parnikoza et al., 2011). This information has led to a paradigm shift in understanding the age of life in Antarctica.

As for the vascular plants of the Antarctic, it can be assumed that there must be a fundamental similarity in their routes and history of distribution in the Antarctic. Their spread to the maritime Antarctic was shown to occur from the mid- to late Pleistocene in the case of *Deschampsia antarctica* É. Desv. (Fasanella et al., 2017) and late Pleistocene in the case of *C. quitensis* (Biersma et al., 2020). As for them experiencing the Last Glaciation maximum, opinions differ: such a possibility is asserted for *D. antarctica* (Fasanella et al., 2017) and ruled out for *C. quitensis* (Biersma et al., 2020).

A generally low genetic heterogeneity was recorded in the Antarctic for both species of vascular plants (Van de Wouw et al., 2008; Androsiuk, 2015; Andreev et al., 2022). Our research found a low level of genetic polymorphism of *C. quitensis* in the studied region.

Previous works have shown that for *D. antarctica*, clusters of geographic distribution in the Antarctic may correspond to isolated genotypes. In particular, *D. antarctica* from the region of the Argentine Islands-Kyiv Peninsula, geographically isolated from *D. antarctica* on the southern coast of Anvers Island, was genetically distinct (Andreev et al., 2022). Biersma et al. (2020) sampled the plant on the Antarctic Peninsula. By the heterogeneity of chloroplast DNA haplotypes and ITS genotypes, two groups of populations are also observed, corresponding to the north of the Antarctic Peninsula (to the south, including samples from the south of Anvers Island) and its south. (This includes two samples from our study area.)

We identified several genotypes of *C. quitensis* in the Argentine Islands-Kyiv Peninsula region, which is a small fragment of its general range at the junction with another geographical region of distribution of vascular plants (the southern coast of Anvers Island) (Figs. 4, 5). Investigation of the first intron length polymorphism allowed us to identify two main groups of *C. quitensis* populations (Fig. 4). One of them includes plants from the southern locations of the studied region – Cape Pérez, Darboux Island. Another group contains

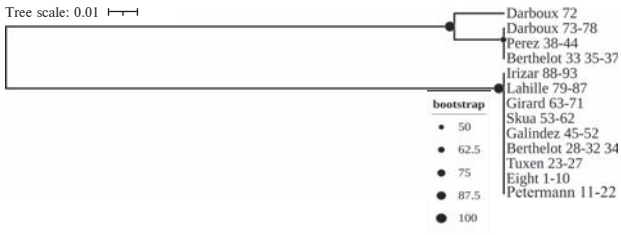

Figure 4. The UPGMA dendrogram based on fragments obtained with the TBP method (first intron of β-tubulin genes) for the 11 *Colobanthus quitensis* populations (93 genotypes). Near the population names, the numbers of analysed samples are indicated

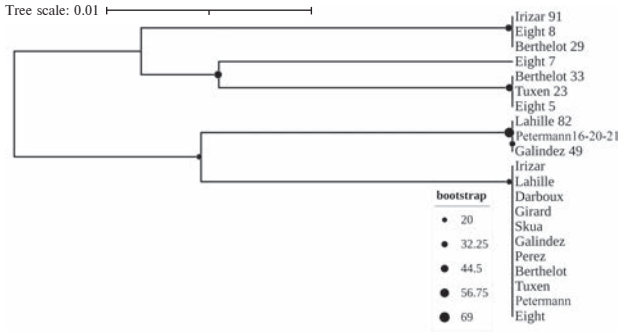

Figure 5. The UPGMA dendrogram based on fragments obtained with the cTBP-method (second intron of β-tubulin genes) for the 11 *Colobanthus quitensis* populations (93 genotypes). Near the population names, the numbers of analysed samples are indicated

samples from all other sites in the studied region. Most of these places, except the southernmost location among the studied locations on Lahille Island, are located north of the previous ones. Unlike to the above groups, the largest of the Berthelot Islands presents plants belonging to both identified groups.

Such findings allow us to assume the possibility of the existence of two variants of genotypes for this marker in the studied region, which probably originate from the north and the south of the Western Antarctic Peninsula coast and are combined in the population of the largest of the Berthelot Islands.

Analysis of the heterogeneity of the second intron β-tubulin genes of *C. quitensis* shows that plants can also be divided into two groups: variable and non-variable (Fig. 5). However, there is no clear geographical pattern. For example, the

plants with variable sequences are present on Galindez, Eight, and Irizar islands but absent on Skua, the island closest to Galindez Island. That the distant and isolated populations of Girard Bay, Skua Island, and Cape Pérez, Darboux Island are genetically similar can probably be explained by the origin of these populations from the representatives of the respective genetic groups brought in there. At the same time, such spreading probably does not occur regularly and is relatively independent of distance. On the other hand, plants from the nearby Cape Pérez and Darboux Island can be assigned to one group. However, according to the results of the analysis of the heterogeneity of the first intron, they also fall into the same group.

Analysing our data, it becomes evident that in the studied region, the borders of both groups of *C. quitensis* plants defined by Biersma et. al. (2020), based on a combination of information on the heterogeneity of chloroplast DNA haplotypes and ITS, also converge. Certainly, given the different methods based on different markers, it is difficult to determine how the variants detected in our study correlate with the variants detected by Biersma et al. (2020) from the northern Antarctic Peninsula and the South Shetland Islands, and once to the southern Antarctic Peninsula. Unfortunately, the region of the Argentine Islands–Kyiv Peninsula is represented in the study by Biersma et al. (2020) by only two herbarium specimens. Nevertheless, our analysis allows us to hypothesize the possibility of the origin of the detected variants based on the variability of tubulin gene introns from different initial locations in the studied region.

What could be the reason for the diversity of *C. quitensis* genotypes? A study using iPBS markers showed that *C. quitensis* populations located at the possible transitional zone – Argentine Islands of the Kyiv Peninsula region are different, which is also consistent with our TBP analysis. The nature of the differentiation probably originated in the starting points of colonization for each variant. Some variability may also be explained by the genome rearrangement caused by mobile genetic elements in response to various stress factors (Androsiuk et al., 2015). According to the concept of the edge of a species range, geographic and peripheral populations of organisms are characterised by low genetic diversity and high rates of genetic differentiation compared to populations from the central parts of the area (Sagarin & Gaines, 2002; Eckert et al., 2008). Thus, the Antarctic biota should demonstrate significant genetic diversity due to the accumulation of genetic mutations and their subsequent fixation in the offspring.

To understand the nature of the genotype variants detected in the region of the Argentine Islands-Kyiv Peninsula, additional studies are needed in the adjacent regions. They may allow us to identify the probable locations from which the detected variants spread to the studied region or to determine which environmental factors could contribute to the accumulation of heterogeneity.

5 Conclusions

Based on the intron length polymorphism analysis of β-tubulin genes, we found a low level of *C. quitensis* genetic polymorphism. It confirms the general situation described in the region. The results of the first intron length polymorphism analysis of β-tubulin genes of *C. quitensis* indicate that among the studied Antarctic pearlwort populations, the populations from Darboux Island, Cape Pérez, and some of the samples from the largest of Berthelot Islands stand out. This may indicate the origin of plants from different regions, while the largest of the Berthelot Islands combines plants from both groups. According to the second intron length polymorphism of β-tubulin genes, most of the studied populations of *C. quitensis* have individual genotypes (in 7 of the 11 studied sites). Such results may also indicate the accumulation of variability in accordance with different environmental conditions at the starting points of the plants' distribution.

Data availability. The data used in this paper is available from the authors.

Author contributions. All the authors have made a substantial contribution to this paper and approved the final version of the manuscript. A.R. performed DNA amplification and prepared the manuscript; Yu.B. wrote the first draft of the manuscript and prepared maps and figures; A.P. performed molecular analyses and data interpretation; L.K. performed data analyses and visualization; I.P. collected samples and contributed to writing the manuscript; I.K. proposed the investigation concept and contributed to writing the manuscript; I.A. contributed to initial analyses and commented on the text; V.K. and Y.P. contributed to writing and edited the manuscript; Y.B. supervised the investigation, reviewed and edited the manuscript.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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Поліморфізм довжини інтронів генів β-тубуліну у *Colobanthus quitensis* в регіоні Аргентинських островів – півострова Київ

Реферат. Генетичні маркери, засновані на оцінці поліморфізму довжини інтронів генів (Intron Length Polymorphism, ILP), в останні роки набули значної популярності в популяційно-генетичних дослідженнях рослин. Один з найпопулярніших ILP-маркерів, що базується на оцінці поліморфізму довжини інтронів генів β-тубуліну різних видів рослин, – TBP (Tubulin-Based-Polymorphism). Цей метод широко використовується для швидкого молекулярно-генетичного аналізу різних видів рослин, адже дозволяє використовувати всього одну комбінацію вироджених праймерів для будь-якої рослинної ДНК. В роботі проаналізовано поліморфізм довжини інтронів генів β-тубуліну в популяціях перлинниці антарктичної (*Colobanthus quitensis*) з відносно компактного регіону Аргентинських островів–півострова Київ: 93 зразки *C. quitensis* з 11 ізольованих острівних популяцій. Результати аналізу поліморфізму довжини двох інтронів генів β-тубуліну в природних популяціях *C. quitensis* загалом свідчать про низький рівень генетичного поліморфізму, що підтверджує загальні уявлення щодо генетичної мінливості *C. quitensis* в Антарктичному регіоні. За даними оцінки поліморфізму довжини першого інтрону виявлено дві групи популяцій *C. quitensis*, що може вказувати на походження рослин з різних регіонів. При цьому ДНК-фрагменти детектуються в діапазоні від 380 п.н. до 1900 п.н., а серед проналізованих популяцій *C. quitensis* виділяються популяції з острова Дарбу, мису Перес та частина зразків з найбільшого з островів Берселот. До того ж Берселот об'єднує рослини з обома типами ДНК-профілю. Оцінка поліморфізму довжини другого інтрону генів β-тубуліну *C. quitensis* дозволила ідентифікувати індивідуальні генотипи у 7 з 11 досліджених популяцій. Отримані результати можуть свідчити про накопичення мінливості відповідно до різних умов навколишнього середовища в початкових точках поширення досліджуваного виду рослин.

Ключові слова: Антарктичний півострів, генетичний поліморфізм, перлинниця антарктична, природні популяції