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## Assessment of *Colobanthus quitensis* genetic polymorphism from the Argentine Islands region (maritime Antarctic) by actin, $\alpha$ - and $\gamma$ -tubulin gene intron analysis

**Abstract.** *Colobanthus quitensis* is one of the two angiosperm plant species commonly spread in the Antarctic. The species has been extensively analyzed at morphological, anatomical and physiological levels, but information regarding its genetic variability remains limited. The aim of the study was to identify molecular genetic differences between *C. quitensis* populations in one of the Antarctic localities, the Argentine Islands region by estimating the intron length polymorphism of actin,  $\alpha$ - and  $\gamma$ -tubulin genes. Samples of *C. quitensis* from different Antarctic natural populations were collected during the season of the 24<sup>th</sup> and previous Ukrainian Antarctic expeditions. Total DNA was isolated using the QIAGEN DNeasy Plant Mini Kit. The polymerase chain reaction was carried out with our own degenerate primers. The resulting amplicons were separated and visualized using polyacrylamide gel electrophoresis followed by silver nitrate staining. Molecular genetic analysis of natural populations of *C. quitensis* was performed using three DNA-marker systems based on the detection of intron length polymorphism of actin,  $\alpha$ - and  $\gamma$ -tubulin genes. A low level of genetic polymorphism of *C. quitensis* in the studied region by these types of markers was established. By assessing the intron length polymorphism of actin genes of the studied *C. quitensis* populations it was possible to establish that the populations of Skua Island had unique amplicons characteristic only for this location. This indicates the possibility of further use of the analysis of intron length polymorphism of actin genes for the study of the molecular genetic diversity of the Antarctic pearlwort. At the same time, the results of analysis of the intron length polymorphism of  $\alpha$ - and  $\gamma$ -tubulin genes induce selection of more specific primers, taking into account the structure of the *C. quitensis* genome. *C. quitensis* in the study region has a low level of genetic variability in intron length polymorphism of actin,  $\alpha$ - and  $\gamma$ -tubulin genes. Overall, the results indicate that DNA markers based on gene structure analysis of highly conserved cytoskeletal proteins, namely, actin,  $\alpha$ - and  $\gamma$ -tubulin, as additional sources of information, can be used for molecular genetic analysis of *C. quitensis* populations in the Antarctic.

**Keywords:** *Colobanthus quitensis*, molecular genetic markers, intron length polymorphism, actin,  $\alpha$ -tubulin,  $\gamma$ -tubulin

### 1 Introduction

The Antarctic pearlwort (*Colobanthus quitensis* (Kunth) Bartl. 1831) and Antarctic hair grass (*Deschampsia antarctica* È. Desv. 1854) are the only angiosperms na-

tive to the Antarctic. The area of *C. quitensis* is quite wide latitudinally (Parnikoza et al., 2018). Isolated island populations of the species occur in a wide range of habitats which might result in the appearance of many and various ecotypes in the region. Besides that,

the reason why the pearlwort's population is growing only in several limited regions of its overall distribution is still a mystery.

*C. quitensis* has been already quite widely analyzed at the morphological, anatomical and physiological levels in order to study its adaptation to harsh environmental conditions (Bravo, Griffith, 2005; Kravets et al., 2011–2012; Gielwanowska et al., 2015; Torres-Díaz et al., 2016). Meanwhile, information on its genetic variability remains so far quite sparse and based mostly on results of only a few studies encompassing a geographically limited set of sampled populations (Lee, Postle, 1975; Gianoli et al., 2004; Acuña-Rodríguez et al., 2014). Recently there came out new reports of molecular genetic analysis of *C. quitensis* dedicated to narrow facets of its genomics, such as the paper on its chloroplast genome (Kang et al., 2016) or the estimate of the genome size using flow cytometry (Cuba-Díaz et al., 2017), while our Polish colleagues (Koc et al., 2018) attempted to evaluate the genetic polymorphism of individual pearlwort plants.

Currently, there is a trend of growing practical use of marker systems built upon estimation of intron length polymorphism (ILP) (Wang et al., 2005; Muthamilarasan et al., 2014; He et al., 2015). Since different organisms have highly conservative sequences of genes encoding cytoskeletal proteins, e. g.  $\alpha$ -,  $\beta$ - and  $\gamma$ -tubulins as well as actin, these genes' introns can be chosen as sound basis for marker system development using their length polymorphism estimates. This type of DNA markers is trustworthy and serviceable since the analysis requires regular polymerase chain reaction (PCR) using primers to exons which flank the introns (Exon-Primed Intron-Crossing (EPIC-PCR)), thus allowing one to use a single combination of degenerate primers for any plant DNA (Badoni et al., 2016). A serious advantage of the method is the fact that the analytical system does not require previous information about specific gene sequences of the studied plant as the primers can be developed for species with sequenced genomes with further application to any other species since the exon sequences of the selected genes are highly conservative in all plants.

Universality, reproducibility and informativeness of ILP-markers have been tested time and again on

various objects. The possibility of applying them to molecular genetic analysis of plants has been examined not only for species but also for cultivars (Breviario et al., 2007; Braglia et al., 2010; Muthamilarasan et al., 2014; Braglia et al., 2014; He et al., 2015; Galasso et al., 2015; Pirko et al., 2016; Rabokon et al., 2019a). The molecular markers of this type have been previously used to reveal and analyze the level of genetic polymorphism of the Antarctic hair grass (Rabokon et al., 2019b).

Since *C. quitensis* and *D. antarctica* have overlapping ranges in the Antarctic, they might have undergone similar stages of adaptation and population spread. Thus a study of genetic differences between the populations of *C. quitensis* in different environmental conditions employing the contemporary palette of molecular genetic markers becomes not merely possible but an urgent task. Application of several ILP-marker systems based on intron polymorphism of genes coding main proteins of the cytoskeleton (actin,  $\alpha$ - and  $\gamma$ -tubulin), can be a valuable tool for further molecular genetic analysis of Antarctic plant species.

**The aim of the study** was to find molecular genetic differences between various populations of the Antarctic pearlwort from the Argentine Island region by evaluating intron length polymorphism of the genes coding actin and  $\alpha$ - and  $\gamma$ -tubulin.

## 2 Materials and methods

### 2.1 Plant material

The study was done using leaves of *C. quitensis* from different natural populations of the Argentine Island region collected during the 24<sup>th</sup> and earlier Ukrainian Antarctic expeditions (Table 1).

### 2.2 DNA isolation and PCR conditions

The samples were collected into sterile plastic containers. The material was frozen until further use and stored at  $-80^{\circ}\text{C}$ . The DNA was isolated using QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instructions. The quality and quantity of DNA was checked electrophoretically in 1.5% agarose gel and spectrophotometrically on "Nanodrop One" spectrophotometer determining the DNA concen-

**Table 1.** Sampling locations of *Colobanthus quitensis* from different populations of the Argentine Islands region

Sampling location	GPS coordinates of the sampling location	Number of samples	Population as marked on Fig. 1–3
Booth Island	S 65.067460°, W 64.015070°	7	H
Eight Island (our proposed working name) from Irizar group, lower locus	S 65.225630°, W 64.209630°	6	D
Skua Island	S 65.255080°, W 64.275060°	6	B
Irizar Island	S 65.219067°, W 64.200167°	3	E
Largest island from the Berthelot Islands group	S 65.328840°, W 64.162580°	5	C
Petermann Island	S 65.165530°, W 64.140150°	2	A

**Table 2.** Description of the primers used

Method	Primers (5'–3')	T, °C
$\alpha$ TBP (I intron of $\alpha$ -tubulin)	F: TGGGARCTNTAYTGYCTYGA R: TCRCTRAARAANGTRTTR AANGMATC	57
$\gamma$ TBP (intron of $\gamma$ -tubulin)	F: GAYGTBTTYTTTTACCARG CKGA R: GAGTTGTARGGYTGGAC RAC	59
ABP (II intron of actin)	F: TGGCATCAYACNTTYTACA AYGA R: CCMCCACTTDAGVACRAT GTT	59

tration and the level of impurities. The DNA samples were kept at  $-20^{\circ}\text{C}$ . Polymerase chain reaction (PCR) was done using ThermalCycler 2720 amplifier ("Applied Biosystems", USA). The reaction mixture (25.0 mkl) contained 2.5 mkl 10x PCR-buffer with ammonium sulfate, 2.5 mM Mg Cl<sub>2</sub>, 50 ng plant DNA, 1 mkM of every primer, 0.2 mM of each dNTP, 0.5 unit Taq-polymerase ("Thermo Scientific", USA). To conduct PCR, we used previously developed degenerate primers (Pirko et al., 2018a, b; Postovoitova et al., 2018b), the sequences of which are given in Table 2.

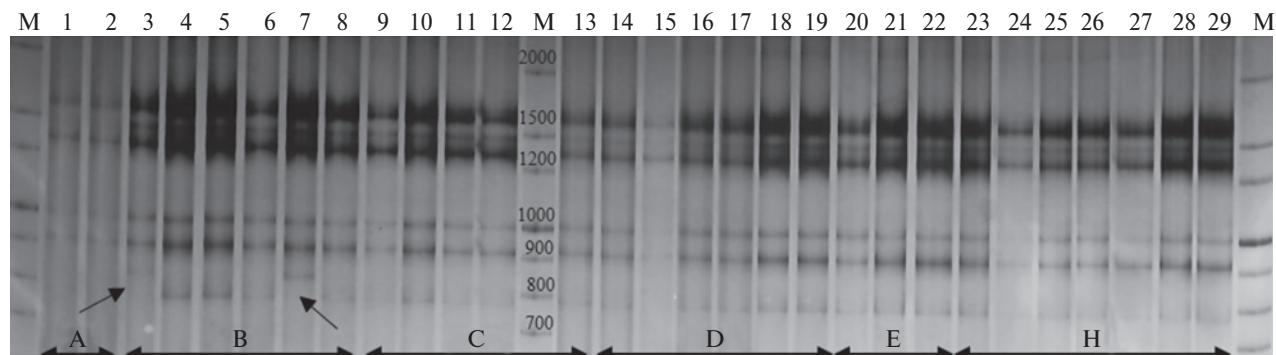
The amplification was done according to the following protocol: initial denaturation ( $94^{\circ}\text{C}$ ) — 3 min, 38 amplification cycles (denaturation at  $94^{\circ}\text{C}$  — 30 s, primer annealing at  $55/57/59^{\circ}\text{C}$  — 40 s, elongation at  $72^{\circ}\text{C}$  — 1.5 min), final elongation at  $72^{\circ}\text{C}$  — 8 min,

$15^{\circ}\text{C}$  — hold. Every amplification reaction was done at least twice employing negative control in order to make it possible to identify non-specific amplification products which would differ in the two tests, in the following electrophoretic analysis. Amplification products (0.5 mkl) were separated using electrophoresis in 6% non-denaturing polyacrylamide gel in 1x TBE-buffer at 380 V for 3 h. Visualization of the DNA fragments was done by staining the gels with silver nitrate. After electrophoresis the gel was photographed in daylight and the images were analyzed. The lengths of reproducible and clear DNA fragments were determined using DNA markers (O'Gene Ruler™ 100 bp Plus DNA Ladder, ready-to-use; "Thermo Scientific", USA) and GelAnalyzer software (<http://www.gelanalyser.com/>).

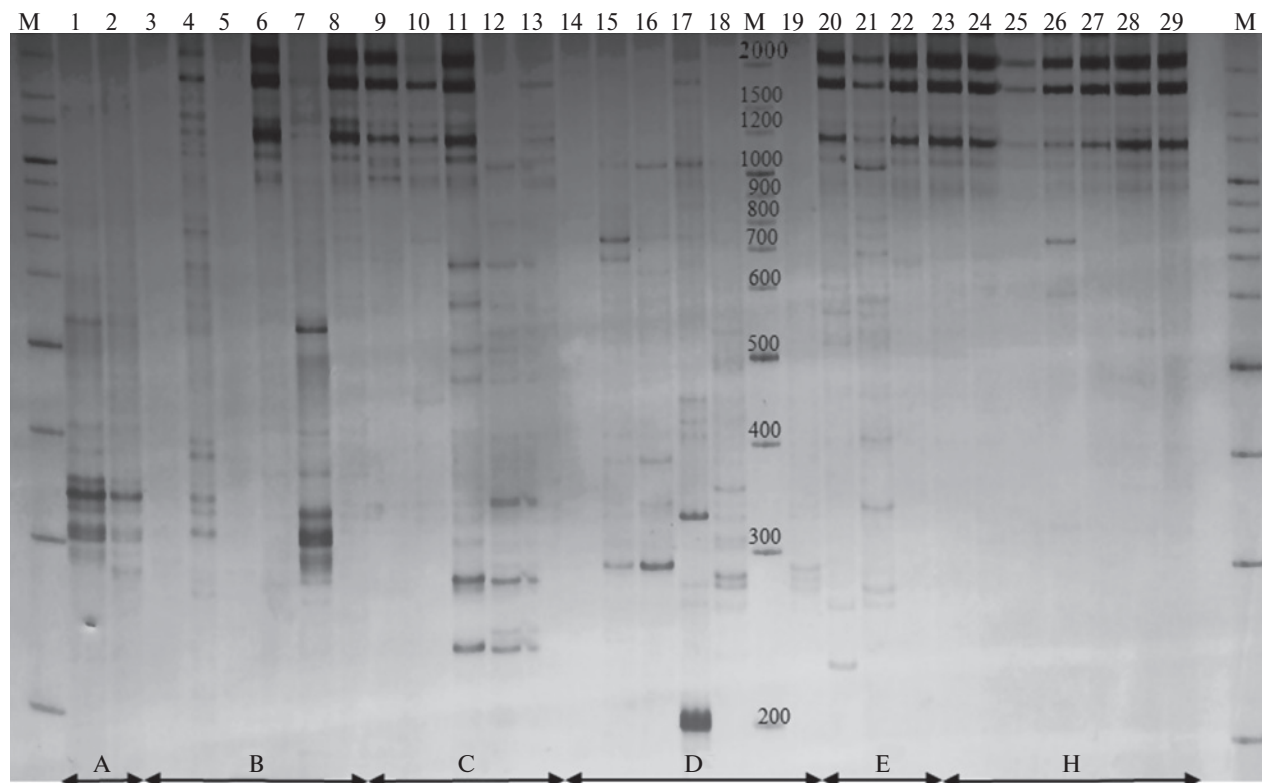
### 3 Results and discussion

#### 3.1 The intron length polymorphism of actin genes in the Antarctic pearlwort

The analysis employing degenerate primers to the introns of the actin gene revealed specific DNA profiles of all studied samples of the Antarctic pearlwort of the Argentine Islands region origin. All in all, the obtained DNA fragments were 700 to 1600 bp (Fig. 1). The range of the fragments lies within expected bounds quite comparable to, and characteristic of other species of plants (Postovoitova et al., 2018a; Postovoitova et al., 2018b). Meanwhile, most of the resulting DNA profiles of *C. quitensis* had six DNA



**Figure 1.** Electrophoregram with actin intron amplicons of *Colobanthus quitensis* from isolated populations of the Argentine Islands region: 1–29 — sample numbers of populations (A — Petermann; B — Skua; C — Berthelot; D — Eight; E — Irizar; H — Booth); M — DNA marker "100 bp Ladder". Arrows indicate zones of polymorphism

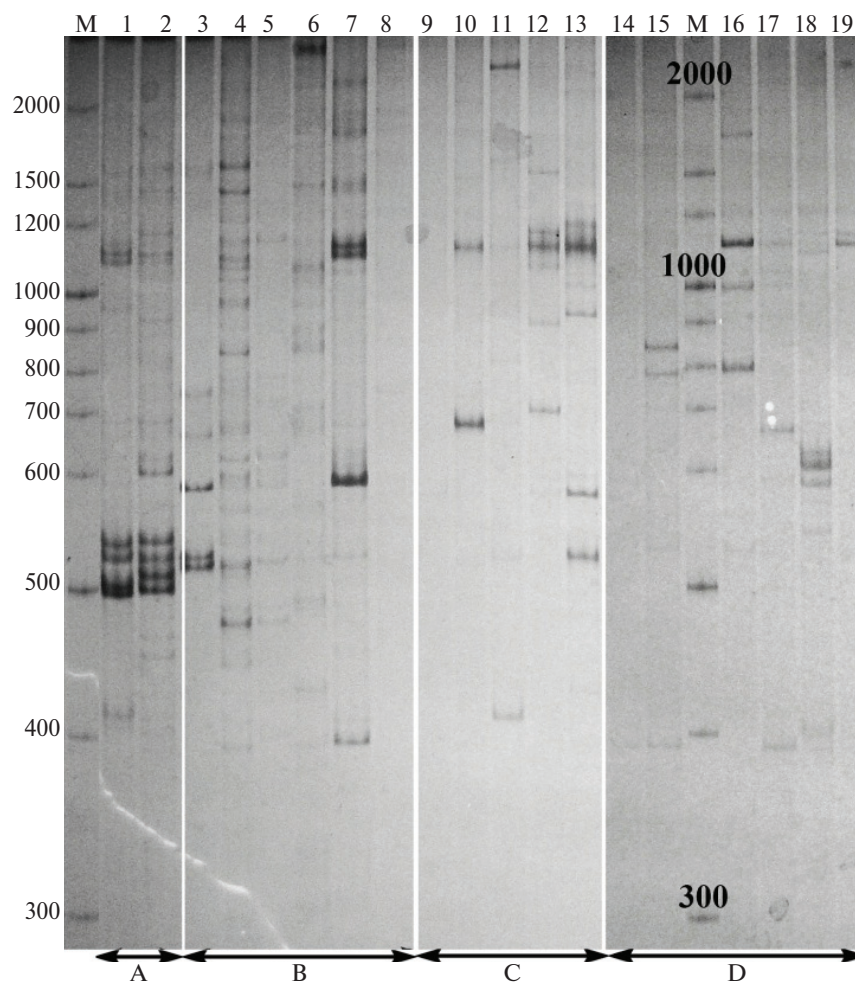


**Figure 2.** Electrophoregram with amplified fragments containing  $\alpha$ -tubulin gene introns of *Colobanthus quitensis* from isolated populations from the Argentine Islands region. 1–29 — sample numbers of populations (A — Petermann; B — Skua; C — Berthelot; D — Eight; E — Irizar; H — Booth); M — DNA marker "100 bp Ladder"

fragments of 791 bp, 909 bp, 1005 bp, 1294 bp, 1495 bp and 1600 bp. It should be noted that samples 3 and 7 differ from all other analyzed for the *C. quitensis* population of Skua Island by having amplicons of

861 bp (Fig. 1, arrows). Such findings allow to hypothesize intrapopulation polymorphism by the marker. From all studies on polymorphism of various genome sequences of *C. quitensis* (Acuña-Rodríguez et al., 2014;





**Figure 3.** Electrophoregram with amplified fragments containing  $\alpha$ -tubulin gene introns of *Colobanthus quitensis* from isolated populations of the Argentine Islands region: 1–19 — sample numbers of populations (A — Petermann; B — Skua; C — Berthelot; D — Eight); M — DNA marker "100 bp Ladder"

Koc et al., 2018; Biersma et al., 2020), only the publication of Biersma et al. (2020) mentions a sample from a nearby region, but it is not Skua Island. Thus, the studied amplicon sample is unique.

On the whole, evaluating the ILP of actin genes did not reveal clear differences between the studied island populations of the pearlwort besides the Skua Island population. However, the data as a whole form a basis for the ensuing application of the DNA marker system to analysis of the ILP of actin genes in order to differentiate on the molecular genetic level the different genotypes of *C. quitensis*. Keeping in mind

that genes of other cytoskeletal proteins such as  $\alpha$ - and  $\gamma$ -tubulin also characteristically contain conservative exon sequences and hypervariable intron ones and can be an important source for genetic polymorphism evaluation (Pirko et al., 2018a, b), we then analyzed their ILP in *C. quitensis*.

### 3.2 Analysis of the intron length polymorphism of the $\alpha$ -tubulin genes in *C. quitensis*

The results of evaluation of the intron length in the genes for  $\alpha$ -tubulin of the pearlwort are given on Fig. 2. Analysis of the distribution of the respective DNA

fragments on the obtained electrophoregram allows us to conclude that they are formed in fairly large quantities with the length varying and not always reproducible. In particular, the electrophoretic analysis detected amplicons in the range of 200 to 2040 bp. Meanwhile most samples (besides samples from Petermann (A) and Eight (D) islands) typically have three intensive high molecular monomorph DNA fragments of about 1190 bp, 1725 bp and 2040 bp. However, it is noteworthy that the samples where the amplicons were found had almost no other amplicons within the expected range (200 to 1000 bp). And vice versa, the samples which had no high molecular products contained amplicons of expected length, for example the sample № 7 from Skua Island (B) — 310 bp, 335 bp, 340 bp and 520 bp. And in some samples (№ 3, № 5, № 14) the amplicons could not be clearly visualized which can be a consequence of several causes, including not precisely adapted PCR conditions.

As mentioned above, the recently developed DNA marker system to evaluate ILP of the  $\alpha$ -tubulin genes has already been applied to other plant species such as *Arabidopsis thaliana* (L.) Heynh. 1842, *Linum usitatissimum* L. 1753, *Oryza sativa* L. 1753, *Solanum tuberosum* L. 1753 and *S. lycopersicum* L. 1753 (Pirko et al., 2018b). It is known that higher plant genomes can have at least 13 genes of  $\alpha$ -tubulin (Radchuk, 2008). However the databases currently lack information not only on the structure of the  $\alpha$ -tubulin genes in the genus *Colobanthus*, but even for any plant of the Caryophyllaceae family, to which the Antarctic pearlwort belongs. Thus, to analyze *C. quitensis* we used degenerate primers to introns developed based on several known sequences of  $\alpha$ -tubulin in plants from other families.

The first intron length of  $\alpha$ -tubulin in plants can vary quite strongly and exceed 1000 bp, for example in the Solanaceae family (Pirko et al., 2018b). Yet the fact that not all samples had high molecular fragments points to the possibility of the hybrid nature of the high molecular fragments, being homo- or heterodimers of smaller DNA fragments. That is the reason why we cannot add the obtained amplicons to analysis. To be certain of the origin of the fragments

and draw conclusions about the genetic structure of the populations of *C. quitensis* based on these ILP markers we would need to carry out further sequencing of the obtained amplicons.

Therefore, the data on the ILP of the  $\alpha$ -tubulin genes of the analyzed pearlwort samples could not be distinctly grouped with the populations of origin.

### 3.3 Analysis of intron length polymorphism in $\gamma$ -tubulin genes in *C. quitensis*

Given that evaluation of the ILP of the  $\alpha$ -tubulin genes did not contribute to the precision of delineation of genetic polymorphism of the studied populations of Antarctic pearlwort, we employed another marker based on the research of ILP of the  $\gamma$ -tubulin genes (Pirko et al., 2018a). The system is easier to use given that usually the  $\gamma$ -tubulin is represented in any eukaryotic genome by only two paralogic genes (Radchuk, 2008), which significantly simplifies the analysis of the obtained results. Besides that, degenerate primers also allow to analyze plants for which there is also no available information on genome structure, as it is for *C. quitensis*.

According to the results of the electrophoretic analysis presented on Fig. 3, it turned out that the Petermann Island samples (population A) produced amplicons within the expected range (500 bp to 600 bp) (Pirko et al., 2018a), and they were similar for samples within a population. For samples from Skua Island (B), largest island from the Berthelot Islands group (C) and Eight Island (D) we also obtained amplicons, yet their number and length were quite different for samples within populations which is not characteristic for introns of  $\gamma$ -tubulin. Firstly, theoretically there should be produced only two fragments since *C. quitensis* is a diploid (Pirko et al., 2018a). Secondly,  $\gamma$ -tubulins are more conservative than other genes of the cytoskeletal proteins, which is why it is unlikely that all samples within every single population can be fully different by its intron length. As to the samples from the Irizar (E) and Booth (H) populations, they did not produce reproducible results. Such results can be only explained by the degenerate primers being annealed not only to the  $\gamma$ -tubulin genes, resulting in numerous non-spe-

cific amplification products. It is also possible that *C. quitensis* has different exon sequences of the  $\gamma$ -tubulin genes than the plants chosen to develop the universal degenerate primers. Evidently, in the future it is necessary to concentrate efforts on developing more specific primers to the exon sequences of *C. quitensis* flanking the first intron of the  $\gamma$ -tubulin gene.

#### 4 Conclusions

Intron length polymorphism analysis of genes coding actin revealed low level of genetic polymorphism in *C. quitensis* by this marker in the studied region. In particular, there was found intron polymorphism of the actin genes at the intrapopulation level among the specimens from the Skua Island population, moreover, the samples had produced unique amplicons not found elsewhere. Analysis of the intron length polymorphism of genes coding  $\alpha$ - and  $\gamma$ -tubulin produced quite a few amplicons of so far unclear origin. We also found that at this point, data on ILP in genes coding  $\alpha$ - and  $\gamma$ -tubulin do not allow efficient genotyping of samples of *C. quitensis* and consequent differentiation of island populations. Yet on the whole the results suggest a possibility of using DNA markers based on analysis of structure of highly conservative cytoskeleton protein genes as an auxiliary tool for molecular genetic analysis of *C. quitensis* populations. For a more streamlined and clear understanding of these results it is advisable to develop species-specific primers to analyze intron length polymorphism in genes coding  $\alpha$ - and  $\gamma$ -tubulin in *C. quitensis* and to conduct additional research encompassing a geographically wider range of habitats.

**Author contributions.** Conception and design: YaP, YaB. DNA extraction: MP, IeP. Sample collection: IP. Development of methodology: AR, AP, YuB, LK, YaP. Analysis and interpretation of data: YaP, IK, YaB. Writing, review, and/or revision of the manuscript: YaP, IK, YaB. All authors have read and agreed to the published version of the manuscript.

**Competing interests.** The authors declare that they have no conflict of interest.

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#### Оцінка генетичного поліморфізму *Colobanthus quitensis* в Антарктиці шляхом аналізу довжини інтронів генів актину, $\alpha$ - та $\gamma$ -тубуліну

**Реферат.** *Colobanthus quitensis* — один з двох видів покритонасінних рослин, поширених в Антарктиці. Хоча цей вид досить широко аналізувався на морфологічному, анатомічному та фізіологічному рівнях, інформація щодо його генетичної мінливості наразі залишається обмеженою. Метою роботи був пошук можливих молекулярно-генетичних відмінностей між різними популяціями перлинниці антарктичної в Антарктиці, шляхом оцінки поліморфізму довжини інтронів генів актину,  $\alpha$ - та  $\gamma$ -тубуліну. В роботі було використано зразки *C. quitensis* з різних природних популяцій Антарктики, зібрані під час експедиційного сезону 24-ї та попередніх Українських антарктичних експедицій. Сумарну ДНК виділяли за допомогою набору QIAGEN DNeasy Plant Mini Kit із дотриманням протоколу виробника. Полімеразну ланцюгову реакцію проводили з власноруч розробленими виродженими праймерами. Утворені амплікони розділяли та візуалізували за допомогою електрофорезу в поліакриламідному гелі з подальшим фарбуванням нітратом срібла. Здійснено молекулярно-генетичний аналіз природних популяцій *C. quitensis* з використанням трьох ДНК-маркерних систем, що базуються на виявленні поліморфізму довжини інтронів генів актину,  $\alpha$ - та  $\gamma$ -тубуліну. Встановлено низький рівень генетичного поліморфізму *C. quitensis* в досліджуваному регіоні за цими видами маркерів. Шляхом оцінки поліморфізму довжини інтронів генів актину досліджуваних популяцій *C. quitensis* вдалося встановити, що популяції з о. Скуа характеризуються наявністю унікальних ампліконів, характерних тільки для цієї локації, що вказує на можливість подальшого використання аналізу поліморфізму інтронів генів актину для дослідження молекулярно-генетичного різноманіття перлинниці антарктичної. В той же час результати аналізу поліморфізму довжини інтронів генів  $\alpha$ - та  $\gamma$ -тубуліну спонукають до підбору більш специфічних праймерів, з урахуванням будови геному *C. quitensis*. *C. quitensis* в досліджуваному регіоні має низький рівень генетичної мінливості за поліморфізмом довжини інтронів генів актину,  $\alpha$ - та  $\gamma$ -тубуліну. В цілому результати досліджень вказують на можливість використання ДНК-маркерів, які базуються на аналізі структури генів висококонсервативних білків цитоскелету, а саме, актину,  $\alpha$ - та  $\gamma$ -тубуліну, як додаткового джерела інформації для молекулярно-генетичного аналізу популяцій *C. quitensis* в Антарктиці.

**Ключові слова:** *Colobanthus quitensis*, молекулярно-генетичні маркери, поліморфізм довжини інтронів, актин,  $\alpha$ -тубулін,  $\gamma$ -тубулін