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GENETIC DIVERSITY IN POPULATIONS OF GENTOO PENGUINS (*PYGOSCELIS PAPUA*)

RAPD analysis was used to examine the extent of genetic polymorphism in two populations of Gentoo penguin (Pygoscelis papua) from Antarctic Islands (Petermann and Livingston). The chosen two of three 10 mer oligonucleotide primers accordingly to preliminary results showed different levels of polymorphism in Gentoo penguins at Petermann Island (from 23,53 to 42,86 %) and Livingston Island (from 52,94 to 57,14 %). Nei's similarity coefficients were in range from 0,5606 (when Gentoo genome profiles were compared with RAPD profiles of two related penguin species: Pygoscelis adeliae (Adelie) and Pygoscelis antarctica (Chinstrep)) to 0,9281 among observed Gentoo penguin populations. Nei's distances values ranged from 0,0746 to 0,5787 among the populations and species. The obtained results will be used for further estimation of genetic diversity of Gentoo penguins and determination of their taxonomic status.

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Introduction. Antarctic ecosystem is relatively new and less influenced by humans then the other ones. Indeed, global warming tendency may influence distribution of plant [1] and animal [2, 3] populations. Thus Antarctic researches which concern distribution of species and their genetic diversity are of fundamental importance.

Penguins are significant part of Antarctic ecsystem and may serve as bioindicator of Antarctica environmental state [4]. Five species of penguins are found on the Antarctic Peninsula and three of them nest (*Pygoscelis adeliae* (Adelie), *Pygoscelis papua* (Gentoo) and *Pygoscelis antarctica* (Chinstrep)) [5, 6]. Penguins are an interesting object for studying genetic diversity and gene flow in populations due to their complicated behavior, ability to migrate and move. Recent work has showed a lack of genetic differentiation between Adelie penguin colonies around the Antarctic continent, despite substantial levels of genetic variation [7]. Some researches also have been dedicated to investigation of phylogeny relationships of penguins [8—10] and changes in widespread and magnitude of penguin populations [11].

But a little is known about genetic diversity in different populations of Gentoo penguin despite their great importance for Antarctic ecosystems. Their total population estimates at 260 000—300 000 pairs. Gentoo penguins nest on rocky coast, sometimes among vegetation. Their nesting grounds tend to shift very slightly from year to year. Little has been discovered about penguins' movements. Gentoo are partial migrants, with subantarctic populations tending to be sedentary, while those of Antarctic Peninsula are migratory [5]. Gentoo penguins are poorly differentiated morphologically, even their sex has to be determined by DNA analysis [12]. So estimation of genetic diversity by morphological characteristics has a little power.

Invention of new PCR-based approaches to test genetic diversity by means of molecular markers gives opportunity to understand better the population genetics of penguins as well as of other species [13]. The aim of the present work was to evaluate the opportunity of utilization Randomly Amplified Polymorphic DNA (RAPD) markers to determine the rate of genetic diversity between populations of Gentoo penguins.

Materials and methods. Source of genomic DNA. 22 tested Gentoo penguin blood samples were collected at Petermann (near Ukrainian Antarctic base Academic Vernadsky and Livingston (near Bul-

garian Antarctic Base «St. Kliment Ohridski») Islands by Ukrainian team in the course of the 8th Antarctic expeditions during summers 2002—2004. Two blood samples of Adelie penguins and two blood samples of Chinstrep penguins were collected at Antarctic Peninsula for comparing RAPD genome profiles. The blood samples anticoagulated with heparin or EDTA were kept at –20 °C. Genomic DNA was isolated by the modified salt extraction method [14]. The phenol-chlorophorm purification with following ethanol precipitation of DNA was performed to avoid inhibition of PCR reaction by heparin. Concentration of DNA probes was determined using spectrophotometer based on absorbance at 260 and 280 nm respectively and was defined more accurately by electrophoresis in 0,8 % agarose gel.

Amplification conditions. RAPD-PCR analysis was carried out as the modified method of Operon. A series of random primers 10 bp each of 60-70 % GC contents (OPA, OPM, OPP (Operon Technologies, Alameda, CA, USA)) and an original primer 11 mer were used for RAPD analysis (Table 1) [15].

A reaction mixture was prepared in 25 ml volume containing 100 ng of genomic DNA, PCR buffer (67 mM Tric-HCl, pH 8,8; 16 mM $(NH4)_2SO_4$; 0,1 % Tween-20; up to 0,1 M β-mercaptoethanol; up to 5 mM $MgCl_2$; 30 pmol primer; 200 mM each of dNTP and 1 U of Taq DNA polymerase (Fermentas). Amplification was performed in AMPLY 4 Biokom programmed for 4 cycles of 1 min 20 sec at 94 °C, 1 min at 40 °C, 2 min at 72 °C; then for 36 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C with the last cycle of 5 min at 72 °C using the fastest available transitions between each temperature.

Table 1

The details of primers along with their sequence and percent (G+C) content used in RAPD analyses of Gentoo penguins

Primer	Sequence 5'-3'	% G+C
11-mer	GGT GGG CTG GA	73
OPP-12	AAG GGC GAG T	60
OPP-14	CCA GCC GAA C	70
OPM-02	ACA ACG CCT C	60
OPP-06	GGG ACG TTG G	70
OPA-06	GGT CCC TGA C	70
OPA-09	GGG TAA CGC C	70
OPA-10	GTG ATC GCA G	60

Agarose gel electrophoresis and visualization of band patterns. Amplified products were analyzed by electrophoresis in 2—2,5 % agarose gel and detected by staining with ethidium bromide [16]. Plasmid pUC 19 digested by restriction endonuclease MvaI and plasmid pUC 19 digested by MspI were used as molecular size standard. A negative control without any DNA template was included in each amplification batch.

Data analysis. RAPD profiles were analyzed by RFLP Scan 3.12. (Scanalytics). Each band was considered as RAPD marker. All the amplifications were repeated two times for analysis and only reproducible bands were considered. We did not count the faint bands. The amplification products of 150—1500 bp were scored across all samples as binary matrix 1 if present or 0 if absent. A band was considered as polymorphic if it was present in at least one genotype and absent in others. Nei's similarity coefficients were calculated using POPGENE version 1.32 [17] (available free from http://www.ualberta.ca/fyeh/). Nei's distances were used as described [13] to quantify the RAPD pattern differences among the stocks and UPGMA was used for cluster analysis using the neighbour program of the PHYLIP package, version 3.63 [18]. Trees were drawn with the treeview program, version 1.4 [19].

Results. *Optimization of primers and RAPD reaction conditions.* Variable concentrations from 20 to 200 ng were used in reaction mixture. One hundred nanograms of DNA gave the most reproducible bands and was thus considered the most ideal and was used subsequently in all analysis. A titration of different concentration of Taq DNA polymerase, $MgCl_2$ and primers revealed 1 U of Taq polymerase, 5 mM of $MgCl_2$ and 30 pmol of primers as the optimum concentrations for PCR amplification.

Seven primers from OPA, OPM, OPP series and 11 mer were screened (Table 1). The primers that produced indistinct amplifications were discarded. Three primers out of 8 were finally selected for analysis of Gentoo penguin populations.

Scoring of amplified products. The number of amplification products was specific to each primer. Primer OPA-10 produced 19 amplified products while primer 11-mer gave 17 products and OPM-

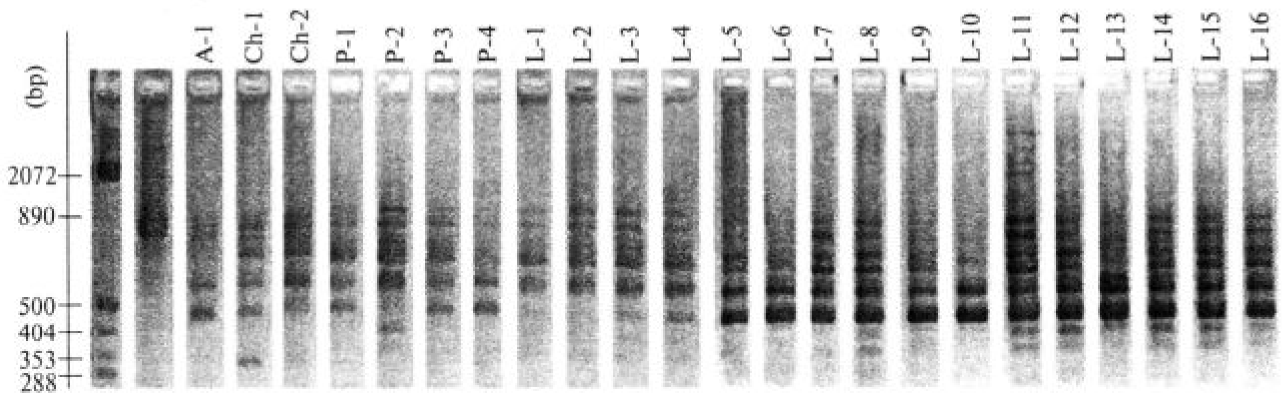

Fig. 1. Electrophoregramm of RAPD-PCR: DNA samples of different penguin species (primer OPM — 02). 1 — molecular weight marker — pUC19 plasmid, digested by MvaI restriction endonuclease and pUC19 plasmid digested by MspI restriction endonuclease; 2 — positive control (890 bp PCR fragment); 3 — DNA of Adelie penguin; 4, 5 — DNA of Chinstrep penguins; 6—9 — DNA of Gentoo penguins (P — Petermann Island); 10—25 — DNA of Gentoo penguins (L — Livingston Island)

02 generated 14 bands (Fig. 1). Primer OPA-10 produced the same polymorphism in two Gentoo populations. Primer OPM-02 and 11-mer produced more than 50 % polymorphism in Gentoo individuals at Livingston Island and medium level of polymorphism in Gentoo penguins at Petermann Island (Table 2).

Estimation of genetic resemblance. So the population of Gentoo penguins at Livingston Island appeared to be genetically more variable when compared to Gentoo penguins at Petermann Island. No fixed specific markers were found for any populations.

Nei's similarity coefficient value [13] ranged from 0,5606 to 0,9281 among the penguins species and populations screened. Nei's distances values ranged from 0,0746 (when we compared Gentoo penguin profiles with RAPD profiles of two related penguin species: Pygoscelis adeliae (Adelie) and Pygoscelis antarctica (Chinstrep)) to 0,5787 among observed Gentoo penguin populations and species screened (Table 3). UPGMA based dendrogram (Fig. 2) constructed by cluster analysis showed that almost all observed Gentoo penguins from two different Antarctic Islands penetrate into the same major cluster (with subdivision into some small clusters) with one exception: individuals L6 and L10 (Livingston). The estimated Fst value was 0,069 indicating that approximately 6,9 % of genetic variability was due to differences between populations. The specimens of Adelie and Chinstrep penguins were grouped into two distinct clusters.

Table 2

Characteristics of primers tested in various populations of *Pygoscelis papua*

Primers	% Polymorphism	
	Pygoscelis papua (Petermann)	*P. papua* (Livingston)
OPA-10	47,37	47,37
OPM-02	42,86	57,14
11-mer	23,53	52,94
Sum of primers	38,00	52,00

Table 3

Nei's measures of genetic identity and genetic distance. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Pop№	Adelie	Chinstrep	Gentoo (Petermann)	Gentoo (Livingston)
Adelie	—	0,5606	0,6289	0,6184
Chinstrep	0,5787	—	0,6826	0,6401
Gentoo (Petermann)	0,4637	0,3819	—	0,9281
Gentoo (Livingston)	0,4807	0,4462	0,0746	—

Discussion. Since it was discovered that DNA fragments could be amplified from DNA of different species which include bacteria [20, 21], plant genomes, endangered species [22, 23] as well as other species, RAPD and other PCR based tech-

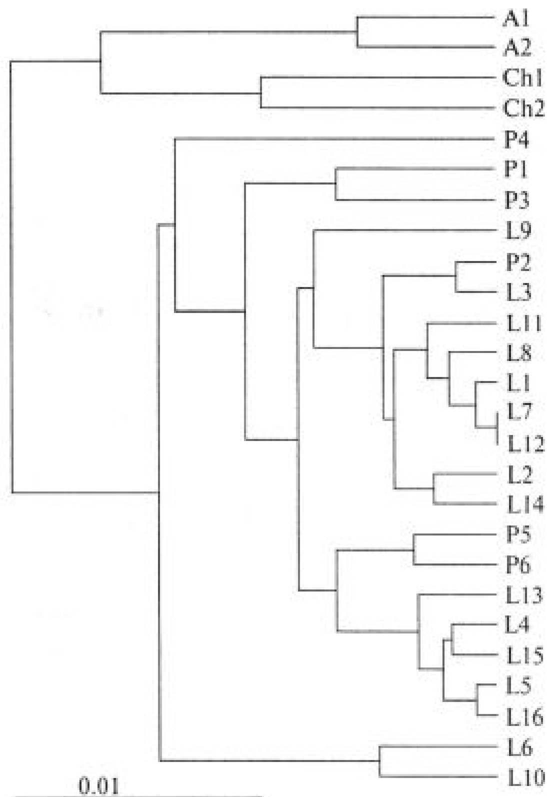

Fig. 2. UPGMA analysis based on RAPD profiles of various Gentoo penguin populations using Nei's distance coefficient. A — Adelie penguins; Ch — Chinstrep penguins; P — Gentoo penguins from Petermann Island; W — Gentoo penguins from Livingston Island. We use the same abbreviations in all text

niques have been used extensively in a wide range of applications [13]. Few studies of terrestrial mammals using the RAPD technique have been reported and different similarity and dissimilarity indexes have been used for RAPD analysis [24, 25]. The lack of standard procedures for RAPD analysis makes comparisons with other studies very difficult [15].

In this study the RAPD procedures proved to be a useful tool for assessing variability because band profiles obtained by three selected primers were reproducible and their patterns of inheritance proved to be Mendelian for a dominant marker.

As already mentioned in this paper Gentoo penguins are very important part of Antarctic ecosystem. They are different from other taxonomic group and provide possible surviving and adaptation in Antarctica. Our main interest has been to define the genetic relationship and genetic differentiation existing between two populations of Gentoo penguins at molecular-genetic level by RAPD markers. Comparing our preliminary results which reflect high level of polymorphism within two penguin populations with data by Nei's coefficients of pair wise similarity, Nei's distance values [13, 26] and UPGMA dendrogram it could be represented the high level of relationship between two Gentoo populations from different Antarctic Islands with a lack of genetic differentiation between them, despite substantial levels of genetic variation [27]. We consider this homogeneity in terms of the dispersal of individuals among colonies and islands. The results are comparable to data obtained by analysis of microsatellites on Adelie and Gentoo penguins [7, 12] and could be due to multiple factors including geographical range (feeding movements among colonies and islands) and ecological niche in addition to genetic drift resulting in change of their genetic patterns by continuous mutations [15]. We found smaller F_{st} value between populations of Gentoo penguins (F_{st} = 0,069) while studies with bird species found the values from 0,17 to 0,20 explaining the result by bird's higher dispersion ability [28]. Accordingly to the results obtained by RAPD analysis of Gentoo penguins from two Antarctic Islands we could confirm that these two populations belong to the same subspecies [12].

Thus, RAPD analysis with three chosen 10 mer primers appeared to be a suitable tool for revealing genetic diversity of Gentoo penguins. Accordingly to preliminary results the primers OPM-02 and 11 mer showed medium level of polymorphism in Gentoo penguins at Petermann Island (from 23,53 to 42,86 %) and high (more than 50 %) polymorphism in Gentoo at Livingston Island (from 52,94 to 57,14 %). Primer OPA-10 produced the same medium level of polymorphism in two Gentoo population (47,37 %). Nei's similarity coefficients were in range from 0,5606 (when Gentoo genome profiles were compared with RAPD profiles of two related penguin species: Pygoscelis adeliae (Adelie) and Pygoscelis antarctica (Chinstrep)) to 0,9281 among observed Gentoo penguin populations. Nei's distances values ranged from 0,0746 to 0,5787 among the penguin populations and species screened. The UPGMA dendrogram grouped Gentoo penguins into one major cluster with a few exceptions. Thus our prior data revealed a lack of

genetic differentiation between two Gentoo penguin populations despite substantial levels of genetic variation. The results obtained by RAPD analysis with chosen primers will be used for further estimation of genetic diversity of Gentoo penguins and determination of their taxonomic status.

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РЕЗЮМЕ. RAPD анализ был использован для исследования уровня генетического полиморфизма двух популяций пингвинов дженту (*Pygoscelis papua*) с Антарктических островов (Питерманн та Ливингстон). Два из отобранных десятичленных олигонуклеотидных праймеров по предварительным результатам выявили разные уровни полиморфизма пингвинов дженту о. Питерманн (от 23,53 до 42,86 %) и о. Ливингстон (от 52,94 до 57,14 %). Коэффициенты попарного сходства Нея варьировали от 0,5606 (при сравнении геномных профилей пингвинов дженту с двумя близкородственными видами пингвинов — *Pygoscelis adeliae* (Adelie) and *Pygoscelis antarctica* (Chinstrep)) до 0,9281 между обследованными популяциями пингвинов дженту. Генетические дистанции Нея составили от 0,0746 до 0,5787 между популяциями и видами. Полученные результаты будут в дальнейшем использованы для оценки генетического разнообразия пингвинов дженту и определения их таксономического статуса.

РЕЗЮМЕ. RAPD аналіз був використаний для дослідження рівня генетичного поліморфізму двох популяцій пінгвінів дженту (*Pygoscelis papua*) з Антарктичних островів (Пітерманн та Лівінгстон). Два з трьох відібраних десятичленних олігонуклеотидних праймерів за попередніми результатами виявили різні рівні поліморфізму пінгвінів дженту о. Пітерманн (від 23,53 до 42,86 %) та о. Лівінгстон (від 52,94 до 57,14 %). Коефіцієнти попарної подібності Нея варіювали від 0,5606 (при порівнянні геномних профілів пінгвінів дженту з двома близькородинними видами пінгвінів — *Pygoscelis adeliae* (Adelie) and *Pygoscelis antarctica* (Chinstrep)) до 0,9281 між обстеженими популяціями пінгвінів дженту. Генетичні дистанції Нея становили від 0,0746 до 0,5787 між популяціями та видами. Отримані результати будуть у подальшому використаними для оцінки генетичної різноманітності пінгвінів дженту та визначення їх таксономічного статусу.

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