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Study of Three DNA Microsatellite Markers in *Pygoscelis papua* Penguins (Aves: Sphenisciformes) from the Livingston Island

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Abstract: In this research we present data from one study of three microsatellite DNA loci in a population of *Pygoscelis papua* penguins, inhabiting the Livingston Island. One hundred and two individuals were genotyped in order to determine the levels of genetic variation in the colony and to evaluate its genetic structure. Our data showed slight allele variations in the studied loci. Two of them were monomorphic and the third demonstrated a low level of polymorphic content with only two alleles presented in the locus.

The obtained data allow us to conclude that the three analysed STR markers appeared to be not enough informative for the investigation of genetic variations within the studied population of *Pygoscelis papua* penguins - this could be due to its very homogeneous genetic structure.

On the other hand, the three microsatellite markers showed to be an appropriate tool for the study of the genetic diversity among the species in genus *Pygoscelis* - for the comparative analyses of the allele frequencies in the studied loci among the 3 species *Pygoscelis papua*, *Pygoscelis antarctica* and *Pygoscelis adelie*.

Key words: The Antarctic, DNA microsatellites, genetic variation, *Pygoscelis papua, Pygoscelis adelie.*

Introduction

The *Pygoscelis papua* Forster, 1781 penguins (Gentoo), together with two other species - *Pygoscelis antarctica* Forster, 1781 (Chinstrap), and *Pygoscelis adelie* Wagler, 1832 (Adelie), belong to genus *Pygoscelis*, family Spheniscidae, class Aves.

The taxonomic status of *Pygoscelis papua* is not clear still. Using morphological evidences, two subspecies were recognized: *Pygoscelis papua papua* and *Pygoscelis papua ellsworthi* (Murphy 1947, according to Metcheva *et al*. 2005), (Stonehouse 1970).

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The subspecies *Pygoscelis papua papua* is considered inhabitant of the sub-Antarctic areas (south to 60º S), *Pygoscelis papua ellsworthii* are considered inhabitants of the Antarctic Peninsula and the South Sandwich Islands - they are with smaller size than *P. p. papua* and proportion of the bills (del Hoyo *et al*., 1992). According to this classification, the studied population, which is from the Livingston Island (South Shetland Islands) (62º38´29´´ S and 60º24´53´´ W), is expected to belong to *Pygoscelis papua ellsworthi* (Metcheva *et al*., 2005).

Pygoscelis papua are the least abundant of pygoscelids, found on the sub-Antarctic islands, with a total breeding populations of approximately 314 000 pairs (Metcheva, Trakiiska 2002), unlike *Pygoscelis adelie*, which are among the most widely distributed species in the Antarctic - more than 10 million birds (Roeder *et al*. 2001).

Recently, the DNA analysis has become widely used in population and evolutionary genetics. There is a shortage of DNA data for the penguins due to the few microsatellite DNA markers available so far.

DNA microsatellites (or STRs - Short Tandem Repeats) are widely distributed in the genome of eukaryotes. Their high variability and selective neutrality make them an extremely useful tool for population and evolutionary studies (Neef, Gross 2001).

Only, one profound microsatellite study in pygoscelids has been reported so far, focused mainly on *Pygoscelis adelie* (about 450 individuals), including the study of 3 individuals of *Pygoscelis papua* and 5 individuals of *Pygoscelis antarctica* as well (Roeder *et al.* 2001). Seven DNA microsatellites were analysed in order to study the genetic differentiation among the *Pygoscelis adelie* colonies around the Antarctic.

In the present study we analysed 3 of these markers: RM3, RM6, TP500, in a representative sample of 102 individuals from one population of *Pygoscelis papua* inhabiting the Livingston Island. The main aim of this research is to determine the genetic variation and to evaluate the genetic structure of the population and to estimate the applicability of the studied STRs in *Pygoscelis papua.*

Materials and Method

The samples were collected from 102 individuals, from a nesting colony in the bay of Kaleta Argentina (62º40´10´´S and 60º24´80´´W), Livingston Island, South Shetland Islands, during the Antarctic summer over the period 2001/2003. The birds were captured by using a hand net. Blood samples up to 1.5 ml were drawn from the cubital vein of the flipper into plastic containers with K_3 EDTA. The samples were placed on ice, transported to the laboratory and stored at -20º C. Total genomic DNA was isolated by phenol-chlorophorm extraction. DNA samples were resuspended in 10mM Tris 0.1mM EDTA pH 7.4 and stored frozen.

Three microsatellites - RM3, RM6 and TP500, isolated from the Adelie's penguin DNA library, were analysed for each sample, using primer sequences described in (Table 1 a,b) (Roeder *et al.* 2001).

PCR amplifications were carried out in a final volume of 10mL, containing 100ng of DNA, 2 pmol of each primer with forward primer, labelled with fluorescent dye Cy5, 1.25mM of each DNTPs, 0.025 units of STS Taq DNA polymerase (Scientific Technologies Sofia), PCR reaction buffer 1x (10mM Tris - HCl, pH 8.3, 50mM KCl), 2.25mM $MgCl_2$ (RM3, RM6) or 1.25mM $MgCl_2$ (TP500). PCR amplifications were performed using Techne TouchGene Gradient.

Table 1. Characterization of the 3 analysed microsatellite loci (number of alleles and their size ranges) in **a)** *Pygoscelis adelie* (Roeder *et al*., 2001) and **b)** *Pygoscelis papua* in our study.

a) *Pygoscelis adelie* (by Roeder *et al*. 2001).

Locus name	Primer sequences 5' to 3'	Repeat sequences of cloned allele	Size range (in bp)	Number of alleles
RM 3	5'-AATCAGGCTCCAAGGTCA 5'-ATGCAAGTGACACAAAGG	$(CA)_{10}$	219 - 231	6
RM 6	5'-CAGGAGGCTTTGAGACAA 5'- CTGTTTACATCCGATGCA	$(CA)_{10}$	168 - 180	6
TP 500	5'- GGACACAGGCAGCCAC 5'- GGGAGTGGTATGGCTGG	$(CA)_{14}$	106 - 126	19

b) *Pygoscelis papua* (our data).

Locus name	Primer sequences 5' to 3'	Repeat sequences of sequenced allele	Size range (in bp)	Number of alleles
RM 3	5'-AATCAGGCTCCAAGGTCA 5'-ATGCAAGTGACACAAAGG	$(CA)_{11}$	217 - 221	2
RM 6	5'-CAGGAGGCTTTGAGACAA 5'- CTGTTTACATCCGATGCA	$*(GT)_6$	168	1
TP 500	5'- GGACACAGGCAGCCAC 5'- GGGAGTGGTATGGCTGG	Non -difined	106	1

* The sequence of RM6 is identical to those published in the GenBank database, but differed from the published by Roeder et al. in Pygoscelis adelie.

The PCR cycles were realized with the following sets of conditions: initial denaturation at 95° C for 5 min, followed by 35 cycles of: denaturation at 95° C for 30s, annealing at 61° C for 30s (RM3), or 64° C for 30s (RM6), or 63° C for 1min (TP500) and elongation at 72° C for 1 min. Final extension was performed at 72° C for 5 min.

By some reasons, locus RM6 was very difficult for amplification. In the beginning of our study of RM6, we met the number of amplification problems, using the set of conditions, given by Roeder *et al.* (2001) - initial denaturation at 94° C for 4 min, followed by 30 cycles, denaturation at 94° C for 45s, annealing at 57° C for 50-60s. It was getting over by a large number of optimization procedures in order to provide optimal PCR conditions – finally it was found the next set of conditions: initial denaturation at 95° C for 5 min, followed by 35 cycles, denaturation at 95° C for 30s, annealing at 64° C for 30s. It was reported by Roeder *et al.* (2002), that RM6 did not amplify in other species: Royal, Humboldt and Emperor Penguins.

The PCR products were analysed in 6% 7M Urea PAA gels using ALF Express sequencer (Pharmacia LKB) at 2000V, 70mA, 45W, 50°C for 180 min. The size of alleles in the studied loci was determined in comparison with two internal standards - 106 bp and 347 bp.

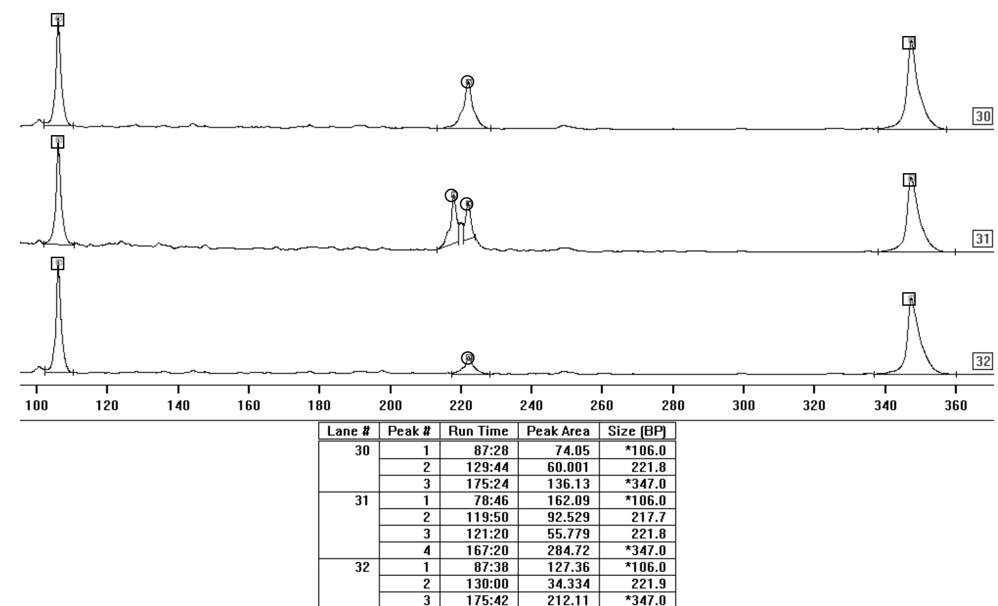
The observed different alleles in the 3 studied microsatellite loci in *Pygoscelis papua* were sequenced on ABI Prizm 310 sequencer (Perkin Elmer).

Allele frequencies at RM3 locus were calculated by simple counting. The observed heterozygosity (Ho) and the expected heterozygosity (Hε) under Hardy-Weinberg proportions were calculated and compared by Fisher Exact Test, using Statistica for Windows (ver. 4.3.), (2×2, Frequency Tables). The comparison between observed and expected (under HWE) genotype frequencies was estimated by χ^2 - method (Statistica for Windows; ver. 4.3.).

Results

The three studied microsatellite loci - RM3, RM6 and TP500, in our sample of *Pygoscelis papua* (Gentoo) population, are dinucleotide short tandem repeats, isolated from Adelie's genome library. The results of our study are summarized in Table 1b.

The RM3 locus contains variable dinucleotide CA repeats. We found 2 alleles (Table 1b), with sizes 217 bp and 221 bp (Fig. 1). In our investigated group the frequency of allele *221* was 0.941 and the frequency of allele 217 was 0.059. Ninety of the analysed individuals were homozygous (221/221). This genotype was calculated with frequency - 0.882. Twelve birds were heterozygous (217/221) with frequency of this genotype - 0.118. Up to now, allele 217 has not been reported to be found neither in *Pygoscelis adelie,* nor in *Pygoscelis antarctica* - the two other members of genus



Lane #	Peak #	Run Time	Peak Area	Size (BP)
30	1	87:28	74.05	*106.0
	2	129:44	60.001	221.8
	3	175:24	136.13	*347.0
31	1	78:46	162.09	*106.0
	2	119:50	92.529	217.7
	3	121:20	55.779	221.8
	4	167:20	284.72	*347.0
32	1	87:38	127.36	*106.0
	2	130:00	34.334	221.9
	3	175:42	212.11	*347.0

Fig. 1. Allele size determination of RM3 microsatellite by sequencing the fragments in ALF Express sequencer is presented. To determine allele size (marked with a circle), the internal standards of 106 bp and 347 bp (marked with squares) were used. The defined alleles are with size 217 bp and 221 bp. Homozygous samples 221/221 bp are shown at lines 30 and 32. Heterozygous sample 217/221 bp is shown at line 31.

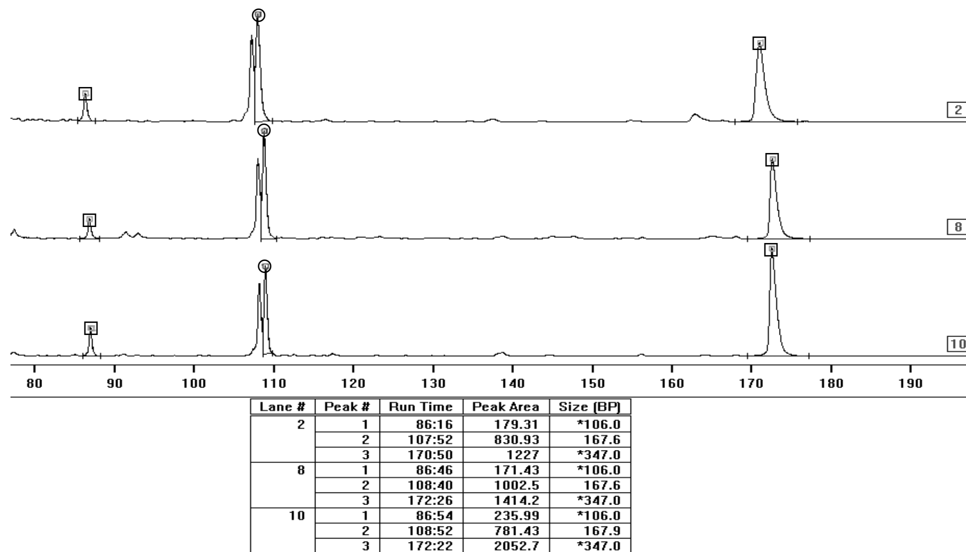
Pygoscelis. Allele *217* was not found in homozygous status, probably because of its low frequency in the group.

The observed heterozygosity (Ho) in the sample was calculated as 0.118 and the expected heterozygosity (Hε) - 0.111 from Hardy-Weinberg equilibrium. The comparison between (Ho) and (Hε) revealed no excess of homozygosity in this locus (HWE; χ^2=0.005; df=1; p=0.82481), (p>0.05).

The observed genotype distribution in the locus showed no deviation from Hardy-Weinberg proportions (HWE; χ^2=0.00453; df=2; p=0.99799), (p>0.05).

Marker RM6 contains dinucleotide GT repeat (Table 1 a,b). In our sample of birds, we found only one allele with a size of 168 bp (Table 1 b), (allele frequency100%), (Fig. 2).

For locus TP500 the data from our study of *Pygoscelis papua* revealed the presence of only one allele with a length 106 bp (allele frequency 100%) (Fig. 3).



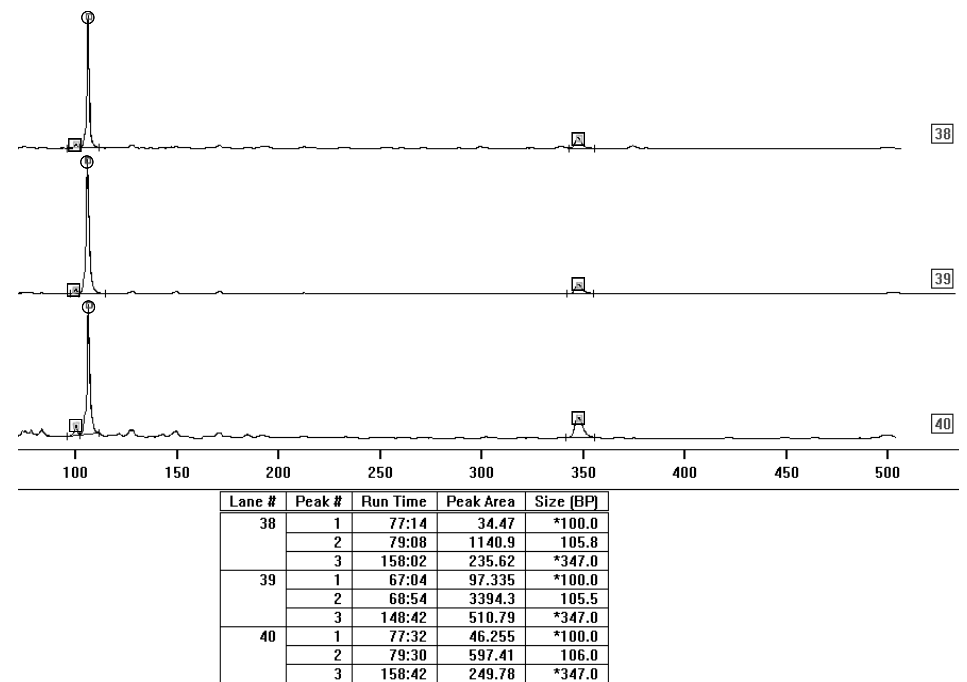
Lane #	Peak #	Run Time	Peak Area	Size (BP)
2	1	86:16	179.31	*106.0
	2	107:52	830.93	167.6
	3	170:50	1227	*347.0
8	1	86:46	171.43	*106.0
	2	108:40	1002.5	167.6
	3	172:26	1414.2	*347.0
10	1	86:54	235.99	*106.0
	2	108:52	781.43	167.9
	3	172:22	2052.7	*347.0

Fig. 2. Allele size determination of RM6 microsatellite, by sequencing the fragments in ALF Express sequencer is presented. To determine the allele size (marked with a circle) the internal standards of 106 bp and 347 bp (marked with squares) were used. The defined allele is with size 168 bp. Homozygous samples are shown at the three lines (2,8,10).

Discussion

Only one profound STR research in pygoscelids has been reported so far, focused mainly on *Pygoscelis adelie*. To date, DNA microsatellite study has been performed only in tree idividuals of *Pygoscelis papua* (Gentoo) penguins (Roeder *et al.* 2001, 2002). We used the DNA microsatellites to analyse the genetic variation and to estimate a genetic structure of the studied *Pigoscelis papua* population.

In this study we determined a new allele - 217 bp, which had not been described for RM3 locus. For some reasons it is possible allele 217 to originate as a consequence



Lane #	Peak #	Run Time	Peak Area	Size (BP)
38	1	77:14	34.47	*100.0
	2	79:08	1140.9	105.8
	3	158:02	235.62	*347.0
39	1	67:04	97.335	*100.0
	2	68:54	3394.3	105.5
	3	148:42	510.79	*347.0
40	1	77:32	46.255	*100.0
	2	79:30	597.41	106.0
	3	158:42	249.78	*347.0

Fig. 3. Allele size determination of TP500 microsatellite, by sequencing the fragments in ALF Express sequencer is presented. To determine the allele size (marked with a circle) an internal standard of 347 bp (marked with a square) was used. The defined allele is with size 106 bp. Homozygous samples are shown at the three lines (38,39,40).

of a new gene flow, but this is not very probable due to the limited size of the studied population (between 50-90 breeding pairs) and its tendency to decrease. Moreover, the penguins show strong natal philopatry, which exclude significant gene flow. Most probably, this allele originates as a consequence of one-step mutation of neighboring alleles, whatever is the most frequent allele in the locus - 221 bp. For this locus Roeder *et al.* reported 6 alleles in *Pygoscelis adelie*, with a length 219 bp - 231 bp. In the samples of Roeder *et al.* (2001), as well as in ours the most frequent allele in RM3 locus was allele 221 bp (0.772). The larger genetic variations at the locus in Adelie pengiuns probably is caused by a much higher number of individuals in Adelie colonies (4000-150 000 breeding pairs) in comparison with the studied Gentoo population (50-90 breeding pairs). We considered that the low number of breeding pairs is probably one of the reasons for the limited genetic variations in the analysed loci.

In our study we determined a total monomorphism in RM6 locus (one allele 168 bp - 100% in a sample). Such monomorphic character of RM6 locus has been reported also for other penguin species: African, Magellanic, Galapagos, Snares crested, Rockhooper, etc. Roeder *et al*. (2001) reported 6 alleles in this locus for Adelie with a length 168 bp - 180 bp. More than one allele was found in Litlle/Fairy (3 alleles), Fiordland crested (2 alleles) and Yellow-eyed (2 alleles) penguins (Roeder *et al*. 2002).

As it was pointed out, our results for TP500 in the studied representative group showed a total monomorphism in this locus - we found only 1 alelle (106 bp - 100% in

a sample). Roeder *et al*. (2001, 2002) reported a very high polymorphic level of the STR locus TP500 in *Pygoscelis adelie* - they observed 19 alleles in Adelie's colonies. The determined length of the alleles in Adelie penguins varied in the range of 106 bp - 126 bp. It is reported as well for the Fiordland crested colony - 6 alleles and for the Rockhooper colony - 5 alleles. In the same study they analysed only 3 individuals of Gentoo and found 1 allele. Despite the high polymorphic level in the other species for TP500, especially in *Pygoscelis adelie*, which exhibited a very high genetic variability, in *Pygoscelis papua* was found a lack of genetic variation in this locus.

The comparisons of the data for all the three markers between Gentoo and Adelie displayed a considerably higher number of genetic variations in *Pygoscelis adelie* for the studied loci, than in *Pygoscelis papua*.

In general, the data of our study in this population of *Pygoscelis papua* revealed a lack of polymorphism in the analysed loci - two of them (RM6, TP500) were absolutely monomorphic, and in the third (RM3) we determined 2 alleles, one of them very rare (allele frequency 6%). These data and the unobserved deviation from Hardy-Weinberg proportions (HWE) could be due to the very homogeneous genetic structure of the studied population.

Although the RM3, RM6 and TP500 markers are not enough informative for study of genetic variation within the population of *Pygoscelis papu*a investigated by us these STR markers are an appropriate tool for study of the genetic diversity among the species in genus *Pygoscelis* - for the comparative analyses of the allele frequencies among the 3 species in a genus for the studied loci.

The finding of more polymorphic and informative microsatellite loci for *Pygoscelis papua* (a construction of *Pygoscelis papua* gene library is needed) as well as the investigations of higher number of new populations of *Pygoscelis papua* and *Pygoscelis antarctica* (data for *Pygoscelis adelie* have been already reported) will allow us to study more profoundly the relationships among the species in genus *Pygoscelis*. It would clarify whether the observed homogeneous genetic structure of *Pygoscelis papua* is due to a very late divergence of the species, or is due to some other reasons in a taxonomic classification.

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Проучване на три микросателитни ДНК маркера при пингвини от вид *Pygoscelis papua* (Aves: Sphenisciformes) от остров Ливингстън

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(Резюме)

В тази статия са представени изследванията върху три микросателитни локуси в една популация на *Pygoscelis papua*, обитаваща остров Ливингстън. С цел да определим нивото на генетичната изменчивост и да оценим генетичната структура на изследваната популация бяха генотипирани 102 индивида. Данните от нашето изследване показаха малко алелни варианти в локусите. Два от изследваните маркери проявиха пълна мономорфност, а при третия локус беше наблюдавано ниско алелно разнообразие (два алела).

Получените данни ни позволяват да направим заключението, че изследваните три микросателитни маркера не са особено подходящи за изучаване на генетичната изменчивост при пингвините от изследваната популация (*Pygoscelis papua*) – много вероятно е това да се дължи на ясно изразената хомогенност в генетична структура, в изследваната от нас колония. От друга страна трите микросателитни маркери показаха, че са подходящи за изследване на генетичното разнообразие в род *Pygoscelis*, а именно за осъществяване на сравнителен анализ по отношение честотата на алелите, определени във всеки от изследваните локуси в 3-те вида *Pygoscelis papua*, *Pygoscelis antarctica* и *Pygoscelis adelie.*