ABSTRACT

Berezkina A.Ye. Population structure and resources of the gastropod mollusk *Nacella concinna* (Strebel, 1908) in the coastal waters of the Ukrainian Antarctic Station "Academik Vernadsky", Argentine Islands archipelago, West Antarctica. Qualification scholarly paper: a manuscript.

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The thesis is dedicated to new data on the population structure of the gastropod mollusk *N. concinna* for the entire island system of the Argentine Islands, West Antarctica. Patterns of limpet distribution in this water area taking into account the morphological and genetic population structure. The phylogeny of the genus *Nacella* and possible regions of origin and distribution are reconstructed. The role of mollusks in Antarctic ecosystems, data on resources and the possibility of its use as an environmental indicator are determined.

Three morphotypes of *N. concinna* with different shell sculptures in the water area of the Ukrainian Antarctic Station "Academik Vernadsky" were identified. The first morphotype has a shell with a smooth glossy dark surface, the second - a classical form with clear radial ribs, the third – with concentric rings and a white top of the shell. It was found that morphotypes do not differ in morphometric parameters, do not have genetic differentiation, which indicates that the three morphotypes of *N. concinna* belong to the same species. The main morphometric parameters were calculated with using non-destructive analysis of the *N. concinna* population in the Wilhelm archipelago water area. The mollusk weight was calculated by the developed formulas. The dependences of length, shell width, mollusk weight, population density on depth, as well as correlations between *N. concinna* population density, shell length and mollusk weight on different transects were established. The absence of visible patterns of mollusk distribution by depth, morphometric parameters of the shell (length, width) and mollusk weight was established. It was shown that Foster's rule regarding the dependence of morphometric characteristics on food resources was not confirmed for

some studied areas of underwater landscapes. The studied transects differ in terms of population density, but the general trend is observed, namely - with increasing depth, population density decreases. Different size classes of mollusks were presented at all investigated depths (1 m, 5 m, 10 m, 15 m) on 8 transects of the Meek Strait, Stella Creek and the waters of Cape Marina Point. The division of the mollusk population into littoral and sublittoral morphotypes has not been confirmed for the Wilhelm Archipelago water area. Different shell sculptures are probably the result of phenotypic plasticity. Obviously, the shell morphology and the weight of *N. concinna* depend on the relief of the bottom, access to food (amount of algae) and wave activity on each studied transect. The studied mollusk inhabits all available underwater landscapes and constitutes a rich biological resource in this area. The calculated biomass of mollusks in the studied area is 1.2 tons (1215 kg) for Meek Channel (9096 m^2), 0.6 tons (642 kg) for Cape Marina Point water area (3982 $м^2$) and 0.43 tons (430 kg) for Stella Creek (1969 $м^2$).

Molecular phylogenetic analysis of *12S*, *16S*, *CO1* and *28S* gene fragments showed that three morphotypes of the Antarctic limpet with different shell morphology belong to the same species of *N. concinna* in the Wilhelm Archipelago water area. Various phylogenetic reconstructions have demonstrated close relationships between *N. concinna* and other nacellids from Patellogastropoda of tropical and temperate waters of the Southern Hemisphere, in particular, species of the genus *Cellana* (*C. capensis*, *C. solida*, *C. taitensis*, *C. pricei*, *C. tramoserica*, and others). It is shown that the nacellids are the autochthons of the Antarctic.

Reconstruction of the Patellogastropoda phylogeny by a fragment of the mitochondrial gene *12S* showed that three morphotypes belong to one species of *N. concinna*. The studied mollusks have a genetic relationship with the other nacellids, in particular the inhabitants of the South American waters and form with them a monophyletic clade with 99 % bootstrap support: *N. clypeater*, *N. magellanica*, *N. deaurata*, *N. mytilina*. *N. clypeater*, *N. magellanica* (from the Chilean coast (Pacific Ocean) and the coast of Tierra del Fuego) form a separate subclade. The genus *Nacella* combines with the sister clade of the genus *Cellana* (99 % bootstrap support), which are inhabitants of temperate and tropical ocean waters. This genus is found in the temperate and tropical Indo-Pacific Oceans, Hawaii, around Australia and New Zealand. The species were also found around the coasts of Japan, the Red Sea, Mauritius, Madagascar, South Africa and the sub-Antarctic islands. The species *C. capensis* inhabits the Indo-Pacific region, mainly off the coast of Australia. *C. solida* is found in the eastern Indian Ocean off the coast of Australia. *C. taitensis* lives along the coasts of French Polynesia and Pitcairn Islands. These two genera form a single monophyletic clade that is sister with a small 54 % bootstrap support to the monophyletic clade which includes species of the genera *Scutellastra*, *Helcion*, *Cymbula*. Representatives of this clade are common in the Atlantic Ocean from the coast of Norway to South Africa. Representatives of the genus *Patella* formed a monophyletic clade with bootstrap 99. The genus is distributed on the Atlantic coast of Europe.

The affiliation of the identified three morphotypes to one species of *N. concinna* is shown by phylogenetic analysis of mitochondrial 16S gene fragment. The studied sample was divided into two first-order sister subclades with bootstrap 100. The first first-order sister clade is divided into two second-order sister subclades with bootstrap 99. The first clade with bootstrap 100 included Antarctic genus *Nacella* and Indo-Pacific genus *Cellana*, which formed the clades of the third order. All three morphotypes of *N. concinna*, denoted as b1, b2, b3, belong to the same species *N. concinna*. The second and third morphotypes are grouped together with *N. concinna*, collected off the coast of Signy Island. *N. deaurata* from the Pacific coast of Chile occupies a basal position in this clade, while *N. magellanica* from the same region is the "youngest" element in this group, along with *N. mytilina*.

Phylogenetic tree reconstruction of the tree *N. concinna* morphotypes on the conservative mitochondrial *CO1* gene fragment, showed that the studied samples belong to one species. All members of the species *Nacella concinna* were combined in a single clade with bootstrap 100. The studied specimens were genetically related to other nacellids such as South American *N. magellanica* and species *N. delesserti*, *N. aff. mytilina, N. kerguelensis, N. macquariensis, N. terroris, N. edgari* from the water area of the sub-Antarctic islands. Other species of the genus *Nacella* have formed separate clades according to their geographical distribution. On the tree we see two large sister subclades with bootstrap 100. The first subclade of the 1st order includes representatives of the genera *Iothia*, *Tectura, Cellana*. Representatives of the genus *Tectura* formed a separate subclade within the *Iothia* clade with bootstrap 85, which requires further phylogenetic and taxonomic studies. It should be noted that the species *Iothia*, *Tectura*, which formed a single clade, show bipolar distribution. The deep-sea species *Iothia megalodon*, which extends from the mid-latitudes of the Pacific coast of Chile to the Beagle Strait, has taken a basal position. The circum-Antarctic species *Iothia emarginuloides* demonstrates a phylogenetic relationship with it. *Iothia fulva* from the North Atlantic coast of the British Isles and Norway forms a sister clade with a previous species, but with a small bootstrap - 55. The sister clade (77 % bootstrap support) with the previous two species is formed by members of the genus *Tectura*. This genus also shows bipolar distribution and is considered synonymous with *Iothia* (*Tectura*) *coppingeri*. *Tectura virginea* is distributed from the North to the Mediterranean, *Tectura fenestrate* - on the Pacific coast of Alaska, *Tectura testudinalis* - in the Canadian Arctic and Greenland. The studied members of the genus *Tectura* form a monophyletic clade with bootstrap 96. The clade "*Cellana*" is monophyletic and includes only members of the genus *Cellana*. Representatives of the genus are widespread on the coast of the Indian Ocean from Africa to Indochina, around Australia and New Zealand and further reach the Japanese islands. The clade "*Iothia*, *Cellana*, *Tectura*" occupies a basal position relative to the clade "*Nacella*". The second sister subclade of the 1st order is formed exclusively by members of the genus *Nacella*. Monophyletic clade "*Nacella*" includes several subclades. The first basal subclade of the 2nd order is formed exclusively by the species *N. kerguelensis*, distributed exclusively in the sub-Antarctic Kerguelen Island waters. The second subclade of the 2nd order consists of the 3rd order subclade, formed by the species *Nacella concinna*, which is common off the coast of the Antarctic Peninsula. The basal subclade of the 3rd order includes representatives from the sub-Antarctic islands - *N. terroris*, *N. edgari*, *N. macquariensis*. This subclade occupies a basal position. The second subclade of the 3rd order includes: a subclade formed by the species *N. clypeater* from the Pacific coast of Chile; subclade formed by *N. deaurata, N. fuegiensis, N. magellanica, N. mytilina* from the coast of South America. *N. mytilina* is noted from the Pacific coast of Chile, Patagonia, the Atlantic coast of Argentina.

Reconstruction of *N. concinna* phylogeny by the conservative *28S* nuclear gene in the IQtree program by the maximum-likelihood method (consensus tree derived from 10,000 generations) based on Bayesian bootstrap based on 56 sequences from the ncbi service. *Lepetella* (a representative of the vetigastropod) was used as an outgroup. Clades of the 1st order were formed with bootstrap 70. One clade included representatives of the genera *Scutellastra*, *Tectura*, *Patelloida*, *Patella* (bootstrap 51). Species of the genus *Tectura* did not form a monophyletic clade. The sister subclade included representatives of the genera *Cellana*, *Nacella* (bootstrap 44). This subclade is divided into second-order clades with a low bootstrap 44, which indicates the antiquity of this process. The two genera *Cellana*, *Nacella* form monophyletic sister subclades of the second order. The *Cellana* clade has a bootstrap 96, the *Nacella* clade has a bootstrap 97, which confirms the monophyletic nature of these genera. Two sister 3rd order subclades "*Nacella*" with bootstrap 94 are formed as follows. The first subclade with bootstrap 95 formed *N. clypeater*, *N. mytilina*, *N. deaurata*, *N. flammea*, *N. magellanica*. *N. clypeater* from the Pacific coast of Chile occupies a basal position in this subclade. Subordinate position is occupied by *Nacella mytilina*, which is common in the waters of Tierra del Fuego, adjacent to the Pacific and Atlantic coasts of South America, the Falklands, Marion (eol.org/page/4793132). Further, the *Nacella deaurata* (eol.org/species/5857969) covers the same areas, the Antarctic Peninsula, as well as most of the sub-Antarctic islands and Campbell Island on the 50th parallel. *Nacella flammea* (eol.org/page/4793148), *Nacella magellanica* formed a monophyletic clade. The first species is distributed on the islands of Tierra del Fuego and the Falklands, and *N. magellanica* in addition to South Georgia and the west coast of the Antarctic Peninsula (eol.org/species/5857967). The sister subclade with bootstrap 94 was formed by *N. kerguelenensis*, *N. concinna*, *N. delesserti*, *N. edgari*, *N. macquariensis* and *N. terroris*. The basal position in this subclade with bootstrap 90 was occupied by *N. kerguelenensis*. Sister subclade *N. concinna* (Antarctic Peninsula) + *N. delesserti*

(Patagonia, sub-Antarctic islands) with bootstrap 100. Sister 3rd order subclade with bootstrap 97 formed by *N. edgari* (Patagonia, sub-Antarctic islands) (eol.org/page/46464990), *N. macquariensis* (Macquarie Islands, Heard, Prince Edward, Campbell, eol.org/page/4793099), *N. terroris* (Campbell Island, New Zealand sub-Antarctic).

Pure bacterial cultures from different morphotypes of *N. concinna* and bottom sediments (from the water area of the Ukrainian Antarctic Station "Academik Vernadsky") were isolated to test the possibility of using the microflora as a phylogenetic marker. Antarctic strains are represented by psychrophilic, mesotolerant, halophilic mostly gram-negative rod-shaped bacteria and cocci. Isolated bacterial cultures have oxidase activity, and some have pronounced agarase activity.

It was shown that the culture fluids of 26 strains (76.4%) (among 34 studied strains) showed keratinolytic activity (KerA), both on the medium with maltose and gelatin as a substrate, and on the medium with the addition of feathers as the main source of carbon and nitrogen.

The highest level of caseinolytic activity was in bacterial isolates from the intestinal tube of mollusks (Skua Creek Strait, from 6 m and 3 m depth) at a temperature of 19° C (0.082 U/ml and 0.027 U/ml, respectively), and 28 ° C (0.074 U/ml and 0.064 U/ml, respectively). The highest level of keratinolytic activity (15 U/ml, 14 U/ml and 8 U/ml) at 19° C was found in cultures isolated from mollusk soft tissues (Skua Creek, 5 m and 3 m, and Marina Point, 5 m), and from the intestinal tube of mollusks (Meek Channel, 8 m and 5 m) (14 U/ml, 7 U/ml, respectively). At 28° C, the highest keratinolytic activity was in bacterial isolates from the intestinal tube and soft tissue of mollusks (Meek Channel, 1 m and 8 m) (9 U/ml and 8 U/ml, respectively). Most often keratinolytic activity was found in cultures isolated from mollusks that were selected from the Skua Creek Strait (6 m, 3 m, 5 m) and the Meek Channel (8 m, 5 m, 1 m). Thus, at a temperature of 28° C more cultures synthesize enzymes with keratinolytic activity (from 1 to 9 U/ml), but at a temperature of 19° C the level of this activity was much higher (from 1 to 15 U/ml). Only 5 bacterial isolates at a temperature of 28° C showed caseinolytic activity at the level of 0.011 to 0.074 U/ml, while at a temperature of 19° C significantly more cultures (10) showed it (from 0, 01 to 0.082 U/ml).

Screening of α-L-rhamnosidase producers among 34 strains revealed activity (from 0.0025 to 0.11 U/mg protein) in 8 strains (23.5%), while it was trace in the culture fluid of strains isolated from the waters of Uruguay (16 m) and Stella Creek (1 m). The maximum α-L-rhamnosidase activity was found in the culture fluid of two strains (0.11 and 0.095 U/mg protein, respectively), which were isolated from the washout of mollusk shells from the waters of Uruguay (depth 16 m) and one strain (0.085 U/mg of protein) isolated from the soft tissues of the same mollusk.

Thus, for the first time we isolated pure bacterial cultures-producers of proteolytic (keratinolytic and caseinolytic activity) and glycolytic (α-L-rhamnosidase) enzymes from *N. concinna* mollusks.

Molecular genetic barcoding of the *16S* gene fragment shows that *N. concinna*-associated microbiota is represented by Proteobacteria (*Pseudoalteromonas*, *Psychrobacter*, *Shewanella*, *Cobetia*, *Psychromonas*), Bacteroidetes (*Bizionia*) and Firmicutes (*Oceanobacillus*).

The systematic position of the Antarctic strains and their probable bipolar distribution have been established. Reconstruction of phylogenetic relationships of mollusk-associated microflora, showed their affinity with bacteria of the Arctic region (*Psychromonas arctica*, *Oceanobacillus picturae*, *Pseudoalteromonas arctica*, *Shewanella vesiculosa*, *Psychrobacter fozi*, *Psychrobacter fjordensis*, *Psychrobacter glaciei* and others), which suggests their bipolar distribution.

Gastropod mollusk *N. concinna* studied in the Wilhelm Archipelago water area. It is both a scientific object of evolutionary biological research and phylogenetic reconstructions, as well as a model object for monitoring ecological transformations under the influence of global climate change. Limpet is a biological resource included in the FAO (Food and Agriculture Organization, United Nations Organization) catalog. *N. concinna* is a genetically heterogeneous species, adapted to a variety of underwater landscapes and capable of widespread settlement. Pure bacterial cultures isolated from mollusks are a potential resource of various cold-resistant enzymes and their subsequent use in industry.

As a result of the research, the taxonomic status and population structure of the genus *Nacella* (from different water areas of the Argentine and adjacent islands) were determined by morphological, molecular genetics and ecological characteristic. The following main conclusions can be drawn as a result of the study.

The division of the mollusk population into littoral and sublittoral morphotypes has not been confirmed for the water area of the studied island system. The distribution of the *N. concinna* population in underwater landscapes does not have clear patterns between the morphometric parameters of the shell, the weight of the mollusk and the depth. In some cases, Foster's rule regarding the dependence of size on energy resources may not be followed.

N. concinna inhabits all available landscapes, forms a population with high phenotypic plasticity, which includes three morphotypes of shell sculpture, and is a rich resource in the study area.

Molecular phylogenetic analysis of mitochondrial *12S*, *16S*, *CO1* genes and nuclear *28S* gene showed that three morphotypes (selected by the shell morphology) belong to the same species *N. concinna* in the Wilhelm archipelago water area, West Antarctica. Phylogenetic reconstructions have shown close relationships between *N. concinna* and the nacellids of Tierra del Fuego and sub-Antarctic islands.

Phylogenetic relationships of nacellids with Patellogastropoda of tropical and temperate Atlantic Ocean waters are shown. It is established that the nacellids are indigenous to Antarctica. The Kerguelen Plateau and the boundary of the Antarctic Peninsula and Tierra del Fuego, which are associated with the ancient Triassic fauna of southern Gondwana, are likely sites of primary speciation by molecular clock.

Molecular genetic barcoding of the *16S* gene fragment showed that the *N. concinna*-associated microbiota belonged to Proteobacteria (*Pseudoalteromonas*, *Psychrobacter*, *Shewanella*, *Cobetia*, *Psychromonas*), Bacteroidetes (*Bizionia*) and Firmicutes (*Oceanobacillus*).

Reconstruction of the phylogenetic relationships of mollusk-associated microflora showed their affinity to bacteria in the Arctic region and the possible bipolar nature of their spread.

It has been shown that the microbiota associated with *N. concinna* cannot serve as a phylogenetic marker of evolutionary processes, but can be used as an ecological marker of underwater landscapes and mollusk subpopulations.

Key words: *Nacella concinna*, Antarctic limpet, mollusk population structure, phylogeny, barcoding, molecular clock, mollusk-associated bacteria, bipolar distribution, bacterial enzymes, West Antarctica