

SKIN MICROBIOME OF FREE-LIVING EUROPEAN POND TURTLE (*EMYS ORBICULARIS* (L.)) ON THE NORTHERN BORDER OF ITS RANGE IN SILENE NATURE PARK, LATVIA

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Microbial organisms play key roles in animal health and ecology. *E. orbicularis* is included in the Latvian list of protected species. The aim of this preliminary was to get knowledge about bacteria of *E. orbicularis* external microbiome. Individual colonies of bacteria were described based on size, color, texture and morphology, optical microscope was used. The bacterial communities largely consisted of two phyla, the Proteobacteria and Firmicutes. Most common found bacteria were *Pseudomonas* spp. (40%) and *Proteus* spp. (27%). This study provides new basic information about the external microbiomes of *E. orbicularis* and is the first step in understanding their environmental roles.

Key words: European pond turtle, skin microbiome.

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INTRODUCTION

Animals harbor diverse assemblages of microbial organisms that play key roles in host health and ecology (Mattoso et al. 2011, Harris et al. 2009, Mao-Jones et al. 2010, April 2017). One of the conservation strategies of threatened animal species is housing them in captivity, but many studies had shown decreased or increased microbial diversity in captive hosts compared to free-living animals due to abnormal diets, artificial and sterile environment conditions, antibiotic treatment, stress etc. Lack or imbalance in composition of hosts microbiome in captivity can negatively affect their health (gastrointestinal,

respiratory, cardiac disorders etc.) and lead to implications for reintroduction schemes. Reintroducing individuals with microbiomes different from wild populations may increase risk of infections, enteric diseases and transmitting abnormal microbiota to wild populations (McKenzie et al. 2017, Moeller et al. 2013). Thus knowledge of host organisms' microbiome may be important for conservation efforts (Buhlmann et al. 2009, Rhodin et al. 2017).

Turtles in general are in increased need of microbiome research, as greater part of all species are endangered or threatened (West et al. 2019, Redford et al. 2012). The European pond turtle (*Emys orbicularis* L., 1758) is a species

of long-living freshwater turtle in the family Emydidae. Latvia is located in the far north of the modern range of the European pond turtle (Pupins et al. 2017). *E. orbicularis* is included in the Latvian list of protected species (Cabinet Regulation No. 396 2000). In 2014 in accordance with the state approved Species conservation plan and in the framework of the LIFE Project LIFE-HerpetoLatvia 42 captive young adult and semiadult *E. orbicularis* were released in wild for 3 populations restoration in Silene Nature Park, Natura2000 territory (Latvia), 15 from them – in the investigated population.

The aim of this preliminary study was to get knowledge about bacteria of *E. orbicularis* external microbiome and how they differ across its body.

MATERIALS AND METHODS

Ethical statement

This work was conducted in accordance with special permission (21/2019-E) for the collecting granted by the Latvian authorities Nature Conservation Agency (NCA) of Latvia.

Sample collection

26 samples from 7 adult *E. orbicularis* (47% from the population) individuals were collected from May to September 2019 in Silene Nature Park NATURA2000 (Latvia) (N 55.690835°; E 26.788760°) (Fig. 1, 2). The turtles were captured by fyke-nets by the NCA certified expert in the category “Amphibians and reptiles”. All the individuals had no visual signs of illness; skin swabs from cloacal, feet and mouth area were taken with sterile Amies swab using a different swab for each area. After sampling, animals were returned to the place of capture. Swabs were placed individually into vials with nutrient media (agar gel) for transportation.

Culturing bacterial strains

For bacteria growth a sterile Petri plate with Plate Count Lab-Agar™ was inoculated with an Amies swab and the sample was dispersed using the streak plate method. Plates were incubated in aerobic atmosphere for 72 hours at temperature of 30°C (Atlas 2004). For isolation and differentiation of bacteria CHROMagar™ Orientation agar was used (incubated in aerobic conditions at 37°C for 24 hours). Identification of

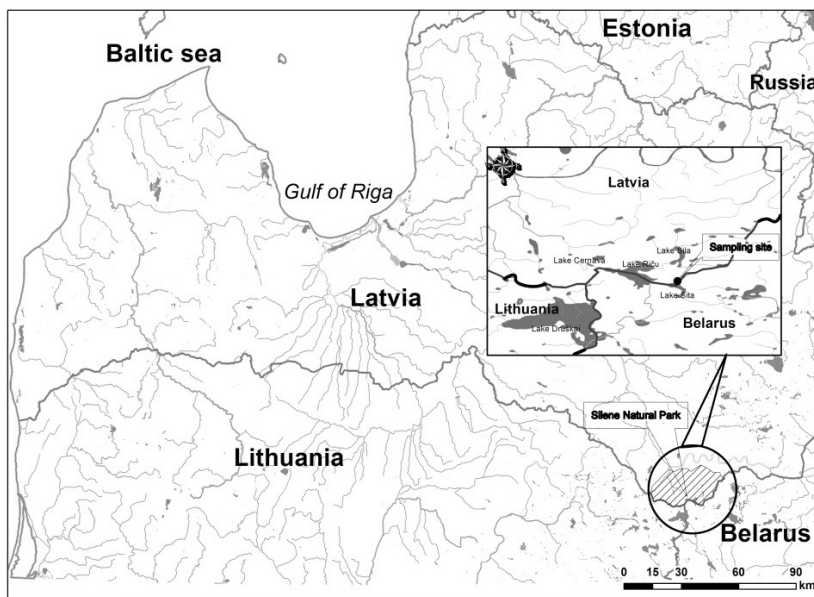


Fig. 1. Sampling site.

bacteria was carried according to CHROMagar™ Orientation Instructions For Use.

Characterization of microbial isolates

Individual colonies of bacteria were described based on size, color, texture and morphology (Cappuccino & Welsh 2016). Gram's staining was performed as described (Coico 2006). Cells are stained with crystal violet, then they are treated with iodine, forming a crystal violet/iodine complex in the cell. Next they are washed with an organic solvent (acetone-alcohol (1:1)), then they are stained with red counterstrain – safranin. Bacteria shape and Gram's reaction was observed under the microscope (Zeiss Axioskop 40 with camera Axiocam 506 Color, magnification – x1000) (Fig. 3). The research method allow to determine bacteria family.



Fig. 2. Sampled turtle with the biotope (Photo: M. Pupins).

RESULTS

The bacterial communities largely consisted of two phyla, the Proteobacteria and Firmicutes. Bacteria found were *Pseudomonas* spp., KES group (*Klebsiella*, *Enterobacter*, *Serratia*), *Proteus* spp., *Staphylococcus* spp., *E. coli* (Fig. 4). Relative bacteria distribution among skin areas were calculated (percent from total found bacteria). *E. coli* was not found in mouth area, but in cloaca and feet area it was 9% from total amount of found bacteria. The most common bacteria in all three studied skin areas (cloaca,

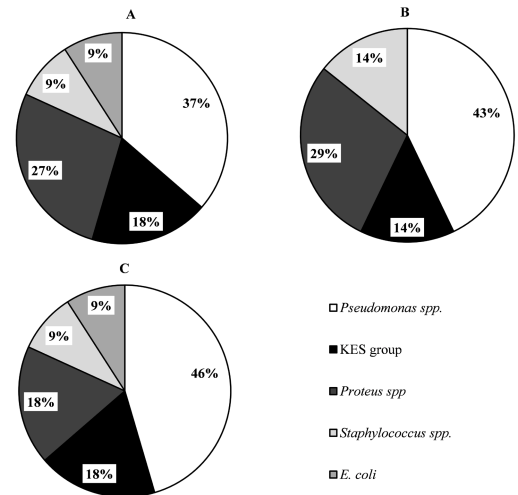


Fig. 4 Microorganisms isolated from the skin surface near the turtle's cloaca (A); mouth (B); feet (C).

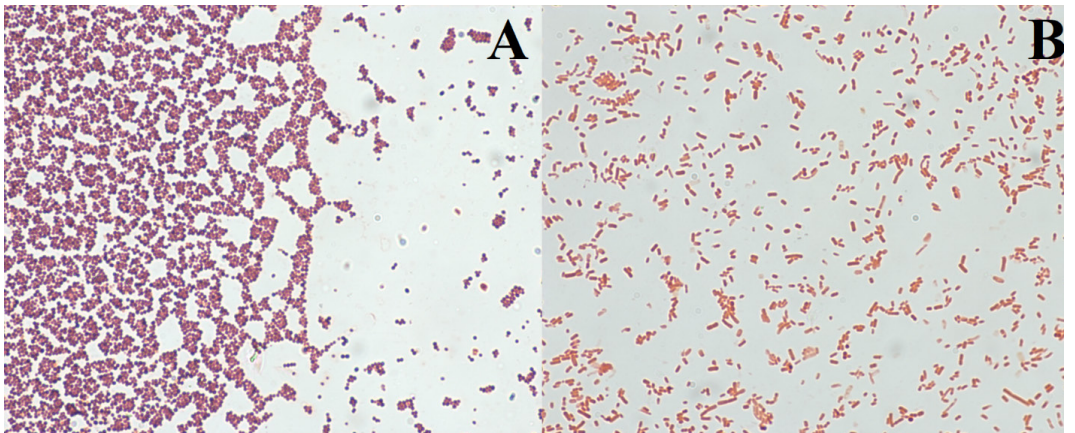


Fig. 3. Gram-positive (A) and Gram-negative bacteria (B) under the microscope. Magnification x1000.

mouth and feet) were *Pseudomonas spp.* (37%, 43%, 46%) and *Proteus spp.* (27%, 29%, 18%) respectively. KES bacteria group in feet and cloaca area was equal (18%), but in mouth it was lower – 14%. *Staphylococcus spp.* were equal in cloaca and mouth area (9%), but in mouth area they were higher – 14%. Bacteria type relative distribution in two skin areas – cloaca and feet, was similar.

DISCUSSION

The bacteria of three different skin areas were examined: mouth area, cloacal area and feet area. This study will provide new basic information on external microbiomes of *E. orbicularis* and is the first step in understanding their environmental roles.

Pseudomonas spp. are Gram-negative, rod-shaped bacteria that inhabits soil and water, and also can cause disease in plants, animals and also humans with immunodeficiency (Wu et al. 2015). It is commonly found in the oral cavity and intestinal tracts of reptiles and can cause a number of diseases such as ulcerative stomatitis, pneumonia, dermatitis and septicemia (Campa et al. 1993, Warwick et al. 2013, Becks & Lorenzoni 1995). In current study *Pseudomonas spp.* bacteria was found in all skin areas (cloaca, mouth and feet) and were the most common bacteria found.

The genus *Proteus* is a Gram-negative bacteria that is widely found in the environment and gastrointestinal tract of animals and human (Hegazy 2016). In animals these bacteria are part of pathogenic or normal microflora (especially in gastrointestinal tract), can be symbiotic or change from neutral/commensal to parasitic (Drzewiecka 2016). *Proteus* is commonly found in cloaca and oral cavity of water turtles and other reptiles (*Mauremys rivulata*, *Natrix natrix*, *Naja atra*, *Thimeresurus albolabris*) (Hacioglu & Tosunoglu 2014, Shek et al. 2009). There is a report of diseases and mortality in sea turtles (Canary Islands, Spain) as well as low egg hatch success rate in loggerhead sea turtles (*Caretta*

caretta) connected with *Proteus spp.* (Oros et al. 2005, Awong-Taylor et al. 2008). In contrast, these bacteria are found as part of normal nasal, cloacal and oral microflora in turtles (Santoro et al. 2006, Foti et al. 2009). In this study *Proteus spp.* were found in all studied skin areas (cloaca, mouth and feet) and were one of the most common bacteria found.

Klebsiella, *Enterobacter*, and *Serratia* (KES group) are closely related gram-negative bacteria, which can be transmitted by contact with wounds or ingestion of contaminated water, feces or other materials. *E. coli* is a common bacterium of intestinal tract of homeothermic vertebrates, isolation frequency from ectothermic reptiles depends on their diet and contact with other animals (Grodon & Cowling 2003). These bacteria have been found in turtles with stomatitis, were cultured from alligators' skin wounds with subcutaneous abscesses, hepatitis and septicemia (Holt et al. 1979, Novak & Seigel 1986, Buenviaje et al. 1994). In current study KES group bacteria were found in all of the studied skin areas. *E. coli* was found in cloaca and feet area, but not in mouth.

Staphylococcus spp. can be a part of healthy oral flora in snakes (Draper et al. 1981), and also as part of pathogenic flora in alligators with respiratory disease, tortoises with stomatitis, sea turtles with lesions of the gastrointestinal tract and hepatitis (Mainster et al. 1972, Holt et al. 1979, Oros et al. 2004). In this study *Staphylococcus spp.* were found in all three studied skin areas.

Found bacteria mostly are opportunistic commensals and can cause diseases only in immunosuppressed, malnourished and wounded hosts. As turtles in this study had no visual signs of illnesses it can be concluded that all found bacteria is part of normal microbiome. The main bacteria in native turtle species require further research to determine the functional role of skin microbes in reptiles and the strength of the environment in the formation of bacterial skin communities.

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