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Method for identification of avian species by eggshell microstructure: preliminary study

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Abstract

It is widely known that birds and their eggs can be identified by their outer characteristics – shape, coloration, spots etc. These features can not be used when only a single piece of eggshell is available (e.g. remains at nests). The aim of this preliminary study was to determine whether microscopy methods can be used for avian species identification by eggshells. Stereo microscope, transmission and reflection light microscope and scanning electron microscope were used to describe eggshell microstructure, empasizing differences among species. Differences in eggshell structure of some Passerine bird species were obrerved in the eggshell mammillary layer by transmission light microscope. However the size of eggshell mammillae was overlapping, and more studies are necessary to discover species-specific structures in avian eggshell.

Key words: avian eggs, eggshell microstructure, identification of species, mammillae.

Introduction

Some research has been conducted on eggshell microstructure in to obtain a method for simple identification of differences among avian species, when only a small piece of eggshell is available. This would allow to identify species of eggs in museum collections, since it does not require destruction of the egg, and also to identify small remains of eggshells in nests (especially cavities), when species identification by other methods is not possible.

Microstructure of the eggshell has been studied with a focus on poultry and these studies are important for commercial purposes in order to improve eggshell strength (Dawkins et al. 2004; Peebles, McDaniel 2004). Most of the knowledge about eggs and eggshells originate from the studies of domesticated birds, but research on wild bird eggs were started only recently (Gosler et al. 2004; Massaro, Davis 2005). However, A.L. Romanoff and A.J. Romanoff (1949) described differences in eggshell microstructure of various species groups. Microstructure as well as pore differences of eggshells of ratite birds were found not to be related to the certain species (Board 1982; Board, Sparks 1991). Traditional identification methods of birds and their eggs are based on morphological analysis (Svenson et al. 1999; Harrison 1975) and there is a lack of avian species identification methods based on only pieces of cracked eggshell.

Formation of an avian egg occurs in female reproductive organs (Romanoff, Romanoff 1949) whereas an eggshell itself is developed in uterus and has several layers (Fig. 1). The growth of the inorganic part of the eggshell initiates from the inner part consisting of organic shell membranes with mammillary cores (Romanoff, Romanoff 1949; Board 1982; Peebles, McDaniel 2004). The first conic layer in the eggshell mineralization stage is formed by growing mammillary knobs (mammillae or cones). When these knobs conjugate, the formation of a palisade layer begins. Mammillary cores are randomly placed on the shell membrane and therefore pores are formed on the eggshell (Romanoff, Romanoff 1949).

The aim of this preliminary study was to determine whether microscopy methods can be used in avian species identification based on eggshells.

Materials and methods

Eggshell samples

Eggshells of closely unrelated avian species for comparison of eggshell microscopy structures included: goose *Anser* sp. (Anseriformes; n = 1), dubbling duck *Anas* sp. (Anseriformes; n = 1), Great Tit *Parus major* (Passeriformes; n = 2), House Sparrow *Passer domesticus* (Passeriformes; n = 2), Chiffchaff *Phylloscopus collybita* (Passeriformes; n = 1), Starling *Sturnus vulgaris* (Passeriformes; n = 2) and Skylark *Alauda arvensis*

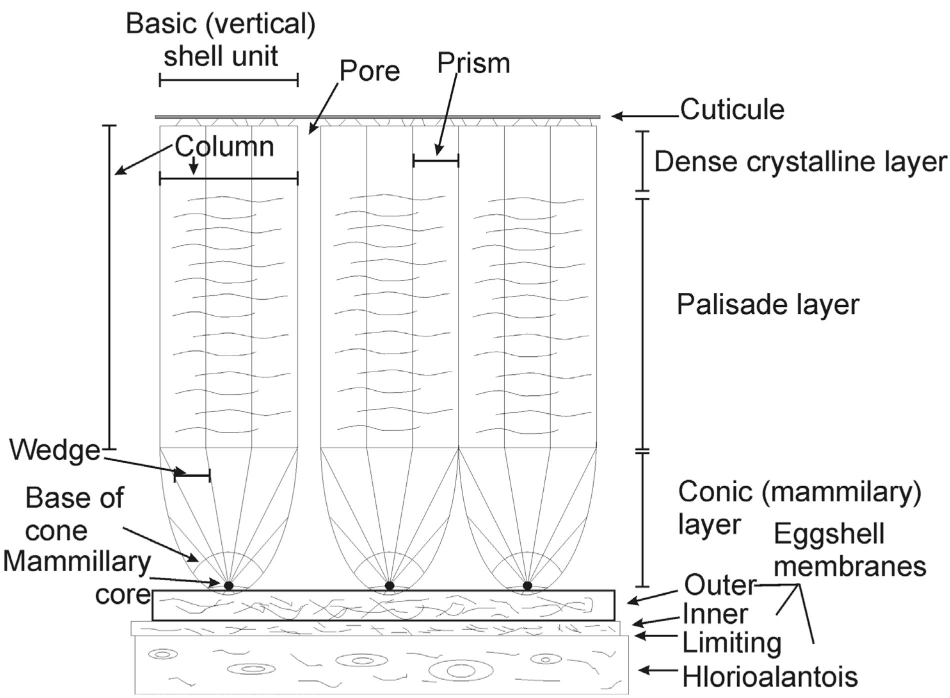

Fig. 1. Structure and elements of the avian eggshell in radial view (Mikhailov 1997; Peebles, McDaniel 2004; Lammie et al. 2005).

(Passeriformes; n = 1). All samples were kindly provided by the Museum of Zoology of the University of Latvia.

Eggshells for analysis of eggshell inner structure were taken from some Passerine bird (Passeriformes) species: Sedge Warbler *Acrocephalus schoenobaenus* (n = 5), Aquatic Warbler *Acrocephalus paludicola* (n = 3), Chiffchaff *Phylloscopus collybita* (n = 3), House Sparrow *Passer domesticus* (n = 5), Tree Sparrow *Passer montanus* (n = 3) and Linnet *Acanthis cannabina* (n = 4). Most of the samples came from collections in the Museum of Zoology (University of Latvia; 11) and Museum of Natural History of Latvia (10). Two of three samples of Aquatic Warbler were collected in Belarus (wild) and Germany (captive population).

Sample preparation and microscopy

Five different microscopes were used in the examination of structure of eggshells: (1) stereo microscope, (2) reflection light microscope, (3) transmission light microscope, (4) fluorescent light microscope and (5) scanning electron microscope.

Stereo microscope. Complete fragments (non ground) of eggshells were examined at magnification of 10 × 0.63 to 10 × 3.

Reflection, transmission and fluorescent light microscope. Leica microscopes were used with magnification of 100, 200 and 400 times. Specimens were prepared in two ways: (1) placing a ground eggshell with water a drop on a glass slide and covering it with cover-slip and (2) placing a non ground eggshell fragment on a glass slide. A digital camera *Canon Powershot S60* (resolution 4 Mpix) was used to take images of the specimens.

Scanning electron microscope (SEM). TM-10000 (*The Hitachi Tabletop Microscope*) was used at magnification of 300 and 1000 times for examination of non ground fragments of eggshells.

The main criteria was detailed investigation were the visibility of as many as possible of eggshell structures in order to compare and determine differences among species.

Analysis of the inner structure of eggshells was performed on a transmission light microscope *Leica DM2000* at magnification 200 times. Samples were prepared using non ground fragments of eggshells. A small fragment of the eggshell (at least 1 × 1 mm) was taken.

The shell membrane that covers the conic layer of the eggshell canblock the view of the inner part of the eggshell. The connection between the membrane and conic layer is very tight (Romanoff, Romanoff 1949), and thus removal of membrane from the conic layer by mechanical methods is impossible. Removal of organic membrane requires chemical methods. Use of acids in this case is not recommended, since calcite reacts with various acids (Brown et al. 2006). Therefore we used 5 % NaOH to remove the membranes (Peebles, McDaniel 2004). Samples were incubated in micro-tubes with 5 % NaOH placed in boiling water bath for 10 to 20 min.

The inner surface of the eggshell was examined. For further analyses images of specimens were taken with a digital camera *Canon Powershot S70* (resolution 7.1 Mpix). Dimensions of mammillae were measured with software *Scion Image for Windows*. Two images of each sample were measured. Minimum (L_{min}) and maximum (L_{max}) size of each mammilla were measured (Fig. 2). Mean size [(L_{min} + L_{max}) / 2] and difference between minimum and maximum size of a mammilla were calculated. For each specimen 33 to 92 measurements of mammillae (59 on average) were made. The number of measurements

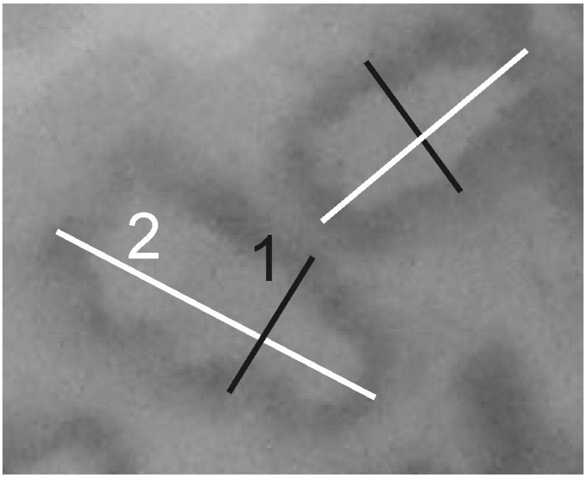

Fig. 2. Measurement of (1) minimum (L_{min}) and (2) maximum (L_{max}) size of eggshell mammilla using a transmission light microscope image at magnification 200 times.

made per specimen depended on quality and size of the shell fragment and quality of the picture. Differences in mammillae dimensions were tested by Scheffe and LSD (Fishers' Least Significant Difference Method) methods using *SPSS for Windows* software (Sokal, Rohlf 1995).

Results and discussion

Comparison of microscopy analysis of eggshell structures

Stereo microscope. No apparent differences were observed among eggshell fragments of various bird species in examination by stereo microscope. Some intra-specific variation was found in spot patterns and structure of the eggshell surface. It was possible to distinguish the inner and outer surface of the eggshell, which is important for identification of the eggshell position when preparing specimens for light microscope.

Reflecting and transmission light microscope. First, a ground eggshell fragment in a drop of water was examined. With reflecting light it was impossible to identify structures that could indicate inter-specific differences. However, observations were obstructed by glare from the cover-slip produced from the reflecting light. Under transmission light it was possible to identify potential variable structures in the ground eggshell substance (e.g. crystallised formations), but identification was impossible. The advantage of this method was the very small amount of eggshell required for the specimen. However, it was impossible to grind eggshells uniformly; hence the consistency of the eggshell was indicated rather than species-specific features. This method needs more exploration, since samples can be obtained with minimal damage to eggs.

Secondly, complete (non ground) eggshell fragments were examined. Differences between inner and outer surfaces of the eggshell were clearly distinguished. The outer surface of the eggshell was considerably glossier than the inner surface. This can be explained by the outer part of the eggshell, palisade layer and cuticle that makes the surface smooth. The inner surface of the eggshell consists of eggshell membranes and the conic layer, which produces irregular structures. Comparing transmittion and reflection light microscopy, a better view was obtained with transmitted light. Reflected light produced a glare that made all structures less distinguishable. Specimens of complete eggshell fragments can be preserved and reused. Thus the procedure can be repeated, if necessary.

Comparing the inner and outer surface of the eggshell among various species, more differences were observed for the inner surface. However, it is known that the outer surface of the eggshell is sometimes covered by cuticle and shell accessory materials that can vary among species (Board 1982; Board, Sparks 1991; Peebles, McDaniel 2004). Evidently, shell accessory materials on the eggshell outer surface are too small to be studied under a light microscope or they are not regularly distributed on eggs. Differences among various species could be observed on the inner surface and are expressed at the conic layer of the eggshell. Eggshell membranes may occur on the conic layer, but differences among these membranes of various eggshells were not observed. This can be explained by the organic origin of the membranes. the conic layer is formed by inorganic material (calcite), and hence it may remain intact for a long time. In this study basal caps of the conic layer were found to be the varying structures of the inner surface – mammillae vary in dimensions, shape, quantity and, probably other parameters. Therefore this feature can be explored further to identify species specific differences of avian eggshell.

Fluorescent light microscope. Some structures of complete eggshell fragments under fluorescent light could be better observed in comparison with transmission light, which might be explained by the luminescent characteristics of calcite (Brown et al. 2006). under fluorescent light, it is possible to clearly separate calcite crystals from other structures in the field of view. However, it was difficult to make qualitative photographs with reflecting light, which limit the possibility to use this method.

Scanning electron microscope. There were similar results found with SEM as shown for transmitting light microscope – there were no clearly visible differences in the outer surface, but it was possible to identify variable structures on the inner surface: mammillae. The advantage of the electron microscope is the high quality pictures that can be produced. SEM has been used for eggshell studies (e.g. Mikhailov 1997), and the acquired images of the conic layer are promisingin the search for differences among species. SEM has been used to measure thickness of the eggshell (Romanoff, Romanoff 1949; Ar et al. 1979; Solomon 1997; Pantheleux 1999; Lammie et al. 2005; Mikhailov 2004; Peebles, McDaniel 2004), detect changes of structure in different stages of incubation (Simons 1971; Hunton 1995; Hunton 2005), observe pore status (open, closed) and dimensions (Board 1982; Board, Sparks 1991). SEM is recommended for further study of the variation of eggshell among species and even individuals (e.g. differences of ecological factors depending on breeding site), nevertheless it is a more complicated method and can not be used out in the field research.

Analysis of eggshell inner structure

Mean mammillae size (minimum, maximum and mean values; Table 1) differed significantly (Scheffe and LSD, $p < 0.04$) between all six studied species (Fig. 3 A - C; Table 2) except between minimum size for Chiffchaff and Linnet by Scheffe (Fig. 3 A; Table 2). The means differed significantly by the LSD test between species of genus *Passer* – Tree Sparrow and House Sparrow, and between these and the other studied species (LSD, $p < 0.01$; Fig. 3 D; Table 2). Using the Scheffe test the significant differences between

Table 1. Sizes and calculated values (μm) of eggshell mammillae

Species	Minimal size	Maximal size	Mean value of sizes	Difference in size	n
Sedge Warbler *Acrocephalus schoenobaenus*	24.23	28.12	26.18	3.89	5
Aquatic Warbler *Acrocephalus paludicola*	26.52	30.27	28.39	3.74	3
Chiffchaff *Phylloscopus collybita*	17.61	20.62	19.11	3.01	3
House Sparrow *Passer domesticus*	31.68	36.74	34.21	5.05	5
Tree Sparrow *Passer montanus*	38.93	44.88	41.91	5.95	3
Linnet *Acanthis cannabina*	18.87	22.88	20.88	4.01	4

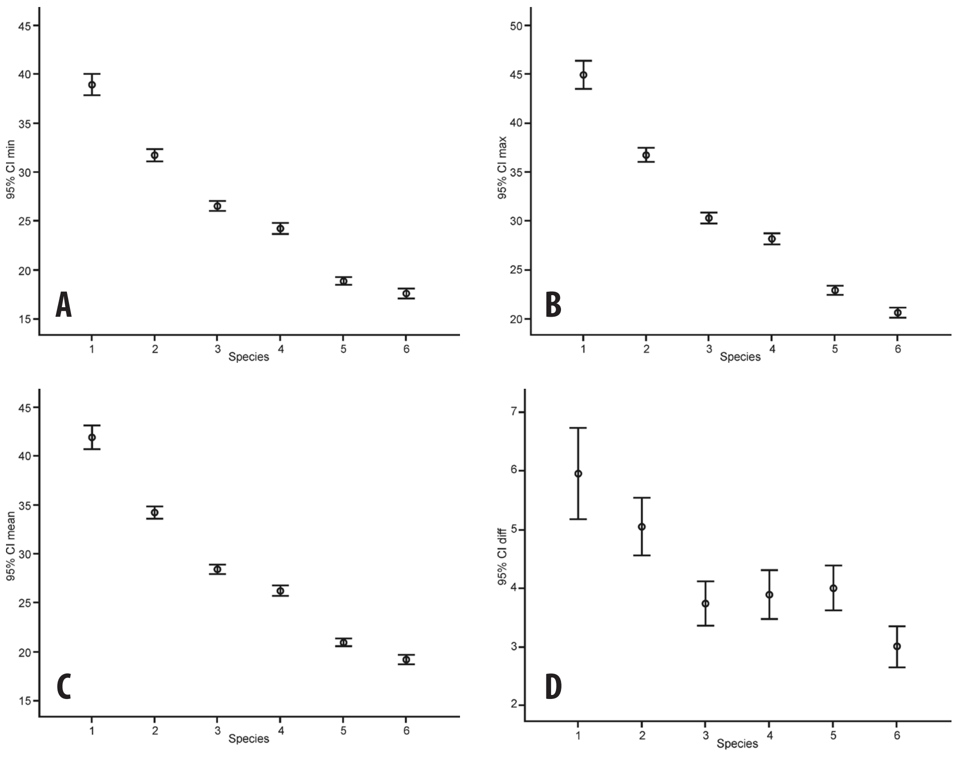

Fig. 3. Size of eggshell mammillae (μm) of the specimens (bars indicate confidence intervals of 95 %). A, maximal size of mammillae; B, minimal size of mammillae; C, mean size of mammillae; D, difference in size of mammillae. Species: 1, Tree Sparrow *Passer montanus*; 2, House Sparrow *Passer domesticus*; 3, Aquatic Warbler *Acrocephalus paludicola*; 4, Sedge Warbler *Acrocephalus schoenobaenus*; 5, Linnet *Acanthis cannabina*; 6, Chiffchaff *Phylloscopus collybita*.

Table 2. Significant differences in sizes of eggshell mammillae by analysis of eggshell inner structure.Data in parentheses are not significantly different

Species		**Tree Sparrow** *Passer montanus*		**House Sparrow** *Passer domesticus*		**Aquatic Warbler** *Acrocephalus paludicola*		**Sedge Warbler** *Acrocephalus schoenobaenus*		**Linnet** *Acanthis cannabina*	
		Scheffe	**LSD**	**Scheffe**	**LSD**	**Scheffe**	**LSD**	**Scheffe**	**LSD**	**Scheffe**	**LSD**
House Sparrow	Min.	<0.001	<0.001								
Passer domesticus	Max.	<0.001	<0.001								
	Mean	<0.001	<0.001								
	Diff.	(0.235)	0.009								
Aquatic Warbler	Min.	<0.001	<0.001	<0.001	<0.001						
Acrocephalus	Max.	<0.001	<0.001	<0.001	<0.001						
paludicola	Mean	<0.001	<0.001	<0.001	<0.001						
	Diff.	<0.001	<0.001	0.005	<0.001						
Sedge Warbler	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
Acrocephalus	Max.	<0.001	<0.001	<0.001	<0.001	0.012	<0.001				
schoenobaenus	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
	Diff.	<0.001	<0.001	0.004	0.001	(n.s.)	(n.s.)				
Linnet	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
Acanthis cannabina	Max.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
	Diff.	<0.001	<0.001	(0.052)	0.001	(n.s.)	(n.s.)	(n.s.)	(n.s.)		
Chiffchaff	Min.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	(n.s.)	0.012
Phylloscopus	Max.	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	<0.001
collybita	Mean	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.036	0.001
	Diff.	<0.001	<0.001	<0.001	<0.001	(n.s.)	(0.059)	(n.s.)	0.029	(n.s.)	0.01

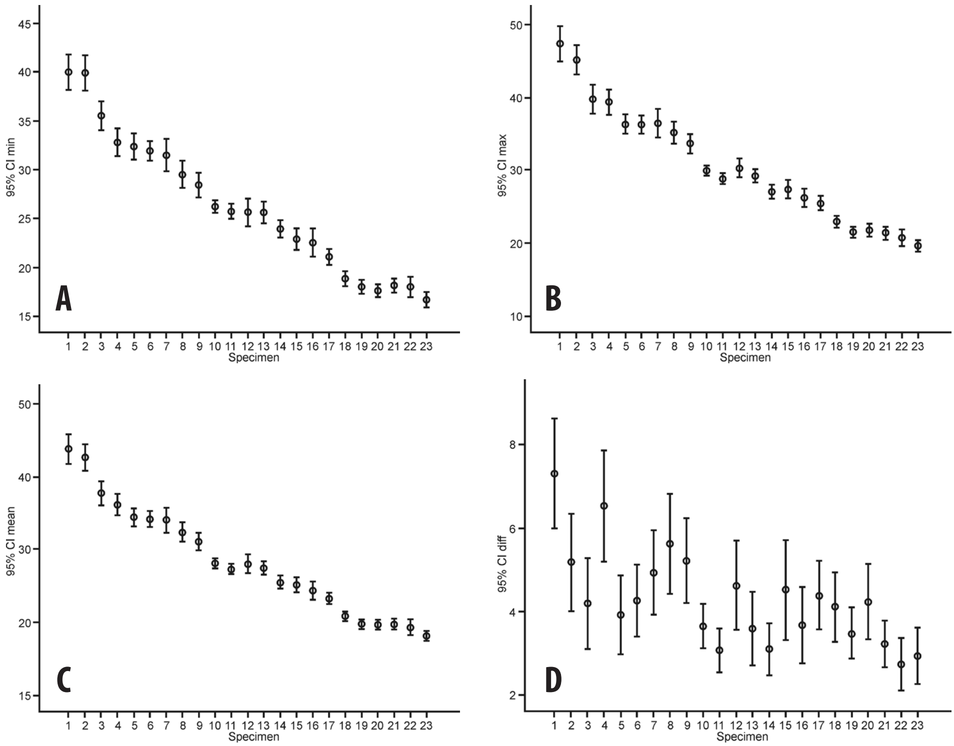

Fig. 4. Size of eggshell mammillae (μm) of the specimens (bars indicate confidence intervals of 95 %). A, maximal size of mammillae; B, minimal size of mammillae; C, mean size of mammillae; D, difference value of size of mammillae. Species: 1 to 3, Tree Sparrow *Passer montanus*; 4 to 8, House Sparrow *Passer domesticus*; 9 to 11, Aquatic Warbler *Acrocephalus paludicola*; 12 to 16, Sedge Warbler *Acrocephalus schoenobaenus*; 17 to 20, Linnet *Acanthis cannabina*; 21 to 23, Chiffchaff *Phylloscopus collybita*.

Tree Sparrow and House Sparrow disappears. Significant differences were observed also between Chiffchaff and Sedge Warbler (LSD, $p < 0.03$; Fig. 3 D; Table 1) and Chiffchaff and Linnet (LSD, $p = 0.01$; Fig. 3 D; Table 2).

The significant species differences largely remained also when any one egg of each species was considered, although in some cases we observed significant variation within one species e.g. Tree Sparrow (Scheffe and LSD, $p < 0.001$), House Sparrow (Scheffe and LSD, $p < 0.001$) and Aquatic Warbler (Scheffe, $p < 0.001$; Fig. 4). However, in some cases there were no differences between samples of different species e.g. we found no significant differences between Sedge and Aquatic Warbler as well as between Chiffchaff and Linnet (Fig. 4).

As significant differences were observed for species of one genus – Tree and House Sparrow this might suggest that phylogeny of the species is not related to size differences of eggshell mammillae. On the other hand, significant differences among samples of one species indicated that size of eggshell mammillae is not species specific. There might be other factors that affect the size of eggshell mammillae (nutrient availability for the female, altitude of breeding site, incubation stage of the egg etc.). It is also likely that a larger sample size for each species might improve the differentiation among species. Some of the eggs at the Zoology Museum of University of Latvia and the Museum of Natural History of Latvia collections were collected in the 1920-ties and 1930-ties and might be misidentified (A. Petriņš, personal communication); thus collection of fresh and correctly identified eggs in the field is necessary for further study.

In the future it is important not only to analyze eggshell structure for species identification, but also eggshell structure in various environments, to exclude the possibility of ecological factors affecting phenological features.

For further studies it is important to explore not only species identification problems, but also eggshell structure in various environments, to exclude the possibility of these factors rather than species causing observed differences in eggshell structure. It can also reveal the intensity of environment and ecological factors affecting eggshell structure.

Conclusions

1. Differences observed in mammillae can be used for avian eggshell analysis.
2. Intra-specific differences of mammillae tends to be significant.
3. Mammillae (minimum and maximum, difference and mean) sizes could not be used for precise species identification and the differences were not species specific, but tendencies of differentiation in species that might be affected by other factors.

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