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Short Communication

Rejection of the synonymization of *Pegosomum saginatum* (Ratz, 1898) Ratz, 1903 with *Pegosomum asperum* (Wright, 1879) Ratz, 1903

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ABSTRACT

The *Pegosomum* Ratz, 1903 are digenean parasites of piscivorous birds. They exhibit few morphological autapomorphies and some of their identification features (number of collar spines) can be altered before or during fixation. Several re-classifications within the genus were suggested, but they have never been supported by molecular analyses. We addressed the synonymization of species within *Pegosomum asperum/saginatum* complex suggested by Dubinin, Dubinina and Saidov. We analyzed one nuclear (ITS2) and two mitochondrial (CO1, ND1) loci of two central European species of *Pegosomum*, namely *Pegosomum asperum* (Wright, 1879) Ratz, 1903 and *Pegosomum saginatum* (Ratz, 1898) Ratz, 1903. Our combined molecular and comparative morphological analyses confirmed the validity of the two *Pegosomum* spp. Both species had highly similar morphology and occurred sympatrically in the gall bladder and bile duct of *Ardea alba* (Linnaeus, 1758). *P. saginatum* occurred more frequently in hosts infected concurrently with *P. asperum*. We also provided host-, age- and sex-specific prevalence and intensity of infections, and comparative measurements of the two *Pegosomum* spp. based on an extensive dataset collected from 1962 to 2016. These species can be morphologically distinguished based on the extent of vitellarium, which reached anteriorly to the pharynx in *P. asperum* but extended anteriorly to the midline of the esophagus in *P. saginatum*. The species also differed in the cirrus diameter (*P. asperum* ≥ 447, *P. saginatum* ≤ 447).

1. Introduction

Based on morphology, two *Pegosomum* spp. appear to parasitize central European birds. These are usually classified as *Pegosomum asperum* (Wright, 1879) Ratz, 1903 and *Pegosomum saginatum* (Ratz, 1898) Ratz, 1903. The two species display limited autapomorphies, only the body size and a body length:width ratio are slightly different, and the two species also differ in the shape of vitellaria in anterior part of the body. Importantly, the differences in body length:width ratios have little identification value in fixed specimens because the pressure applied when the samples are mounted, can alter these values. Several studies, including Dubinin and Dubinina [1] and Saidov [2], suggested that *P. asperum* and *P. saginatum* should be considered as a single species, assuming that the morphological differences are due simply by intraspecific variability. If such differences were a result of intraspecific variation in size of lateral parts of the body adjacent to vitellaria, the intermediate forms should exist [3]. However, such intermediate forms have never been reported. Neverheless, Aleksandrova and Podgornova [4] found that both species have identical karyotypes, with a diploid set consisting of 20 chromosomes of mutually similar morphology and size of bivalents. It is likely that *P. asperum* and *P. saginatum* evolved sympatrically, with later differentiation of infection locations within hosts [4–5]. Despite uncertainties in diagnostic characters of *Pegosomum* spp., there are no molecular phylogenetic data available to date.

In central Europe, previous studies reported *P. asperum* from gall bladders of *Ardea cinerea* and *Ardea alba* in the Czech Republic [6–8], and *P. saginatum* from gall bladders of *A. alba* in the Czech Republic [6–7]. In our previous epidemiological study [8], we noted that the specimens found in *A. alba* could represent either one or two species, thus we indicated only *Pegosomum* sp. infected this host. In addition, Gundlach [9] found *P. spiniferum* (*P. asperum*) in *Ciconia ciconia* in Poland and, over a century ago, István Rátz identified type specimens of *P. spiniferum* (*P. asperum*) in *Botaurus stellaris* and *P. saginatum* in *A. alba* in Hungary [10].

In this study, we used a combined molecular and comparative morphological approach to address the possible synonymization of central European species within the *Pegosomum*, initially suggested by Dubinin and Dubinina [1] and Saidov [2].

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2. Material and methods

To assess the prevalence of infections, we examined over 17,000 individuals of 240 bird species for the presence of helminths of the *Pegosomum* in years 1962–2016 (det. & coll. Sitko). We collected all examined birds in the Czech Republic (48°39′N–50°59′N, 12°19′E–18°29′E), primarily in the eastern parts of the country (Table S1). All helminths were obtained from birds that were deposited in the Comenius Museum in Přerov, Czech Republic. All birds were already dead from various causes when the museum received them.

For the phylogenetic analyses, we examined ethanol-fixed individuals of the *Pegosomum* obtained from the core host, *Ardea alba* in the Hustopeče nad Bečvou (49.57°N, 17.44°E) environments from Mar-2014 to Mar-2016 (Table S2). For comparative morphology, we stained well-preserved individuals from sacrificed hosts in Semichon's carmine, dehydrated by alcohol series, and mounted in Canada balsam. We measured the longest egg present within each adult to analyze egg length. Dimensions were shown in μm as ranges (mean ± SD). All other data were shown as the mean ± SD, unless stated otherwise.

We extracted and amplified DNA as described [11], using primers targeting the nuclear ITS2 locus, and the mitochondrial CO1 and ND1 loci (Table S3). We purified the PCR products using USB Exo-SAP-IT (Affymetrix, Santa Clara, CA) and subjected them to bidirectional Sanger sequencing on an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA). We submitted the resulting consensus DNA sequences to GenBank under accession numbers KX097824-KX097870 (Table S2). Details of the phylogenetical analyses are provided in the Supplementary Appendix.

3. Results

We found only two *Pegosomum* spp., *P. asperum* and *P. saginatum*, in the examined birds. Maximum likelihood analyses of both nuclear (ITS2) and mitochondrial (CO1 and ND1) DNA loci revealed that both *P. asperum* and *P. saginatum* represented distinct, well-defined *Pegosomum* species (Fig. 1A–C). All three hypervariable DNA loci tested (ITS2, CO1 and ND1) were species-specific, differentiating *P. saginatum* from *P. asperum* collected from multiple host individuals. All three loci displayed intraspecific variability; in particular, both mitochondrial loci were highly variable (Table S4). Notably, the intraspecific divergence of each of the three loci tested was five times lower than the divergence between *P. asperum* and *P. saginatum* (Table S4). The NCBI Blast algorithm confirmed the placement of Pegosominae within Echinostomatidae, which was suggested, e.g., by Kostadinova [12]. Despite limited availability of echinostomatid sequences of most of the loci tested, the most similar sequences belonged to *Isthmiophora hortensis* (Asada, 1926) (ITS2), *Fasciola hepatica* Linnaeus, 1758 (CO1) and *Echinostoma* sp. (ND1). Respective loci of these species were thus used as outgroups in phylogenetic analyses. We corroborated the data obtained with maximum likelihood analysis by inferring the tree topologies using a Bayesian approach. Outcomes of the Bayesian approach and of the maximum likelihood analyses were highly congruent (Tables S5–S7).

We found both *Pegosomum* spp. only in herons, with *Ardea alba* considered a core host and *Ardea cinerea* considered a satellite host (Table S8). The prevalence of *P. asperum* in *A. alba* differed only slightly with respect to whether host birds were juveniles in first calendar year rather than sub-adult or adult (27% vs 43%; χ^2 test $p = 0.04$). Differences in prevalence between the sexes of host birds reached also borderline significance (χ^2 test $p = 0.05$), with males being infected more frequently (50%) than the females (31%) (Table S8). Similarly, we repeatedly found *P. saginatum* in both juveniles and (sub)adults and in both sexes. Total prevalence of *P. saginatum* in the core host reached 15%, and this species was absent in other herons and egrets examined (Table S8). Six of the seven herons infected with *P. saginatum* were co-infected with *P. asperum*.

Intensity of infection was significantly higher in *P. asperum*, where it reached 8.1 ± 6.4 individuals per host compared only to 2.1 ± 0.7 individuals of *P. saginatum* per host (*t*-test $p = 0.01$, $n_1 = 145$ *P. asperum* in 18 host individuals and $n_2 = 15$ *P. saginatum* in 7 host individuals). Intensity of infection did not differ between juveniles and (sub)adults and between adults of different sexes (Table S9). Co-infections did not affect the intensity of infection (Table S10).

As *P. asperum* and *P. saginatum* were not subjected to previous DNA analyses, we complement here the DNA analyses with measurements of key features (Table 1, Tables S11–S12) and provide representative drawings (Fig. 1D–E) of both species. The body measurements of the two species overlap with each other. The only classical identification feature consists of the extent of vitellarium. In both species, vitellarium is strongly developed and composed of large follicles. In *P. asperum*, the vitellarium extends anteriorly to the pharynx, whereas in *P. saginatum*, the vitellarium extends anteriorly only to the midline of the esophagus. Numerous other body measures differ significantly between the two species, but their ranges overlap (body length and width, pharynx length and width, esophagus width, bursa cirri length, length of the lateral collar spines, *t*-test $p < 0.001$ each) or are difficult to measure (widths of the medial and lateral collar spines). In addition, the size of collar spines is subject to growth with increasing body size even in the adulthood. Interestingly, we found only a marginal overlap in cirrus diameter between the two species (447–714 vs. 108–447) (Table 1, Tables S11–S12).

4. Discussion

The *P. asperum/saginatum* complex was previously suggested to be one species [1–2]. The only systematic studies of *Pegosomum* spp. taxonomy are several decades old, represented by Aleksandrova [5] and Feĭzullaev et al. [13]. Thus, classification of *Pegosomum* spp. was based only on morphological characters; molecular phylogenetic analyses have not been previously conducted. Analyses of the newly obtained DNA sequences confirmed classification of *P. asperum* and *P. saginatum* as two valid species (Fig. 1A–C). In addition, analyses confirmed the placement of Pegosominae within Echinostomatidae, suggested previously [12].

The majority of prior studies provided narrower ranges for the particular morphological features (Table 1, Tables S11–S12). All sizes overlap completely or nearly completely with the ranges provided in this study with the following exceptions: Dawes [14] provided an erroneous number of collar spines (20 − 21) for *P. saginatum*, and likely took over this information from Rátz, who also claimed the same error. Nevertheless, the complete number of spines for this species should be 27. The collar spines may vary in number and arrangement, and they often detach easily and spontaneously, particularly from the material obtained from the birds found dead ([13]; Sitko, pers. obs.). Thus, any descriptions of *Pegosomum* spp., in which the key identification feature is the number and position of collar spines, should be taken with caution, if not rejected at all. For *P. saginatum*, Aleksandrova and Podgornova [4] and also Aleksandrova [5] suggested a different body length:width ratio, which is caused most likely by applying a different pressure during the preparation of slides. Some authors claim that oral sucker is absent [14]. We disagree with this statement. Oral sucker is present in both species; it is terminal, very small, and poorly visible in fixed individuals. Terminal position of oral sucker causes problems with the identification of its dimensions at fixed individuals. Also ventral sucker of *P. saginatum* is subject to some controversies, as the original description by Rátz [15], and the identical dimensions mentioned by Dawes [14] without citing Rátz, are by 15–41% larger than those reported by us in this study and by Sitko [7] (Table S12). Such difference can be explained by the application of strong pressure during fixation by Rátz, which was common at the end of 19th century, and which can cause the increase by 20% or more (Sitko, pers. obs.). The ranges of the largest eggs found in each examined individual were much broader than thought previously (Tables S11–S12). The body length:width ratio