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Corresponding author:
P. Heneberg;
Email: petr.heneberg@lf3.cuni.cz

Molecular phylogenetics provides unequivocal support for reclassifying *Cathaemasia hians longivitellata* and *C. h. hians* (Trematoda: Cathaemasiidae) as two valid species with different host preferences

Petr Heneberg[1] and Jiljí Sitko[2]

[1]Charles University, Third Faculty of Medicine, Prague, Czechia and [2]Comenius Museum, Moravian Ornithological Station, Přerov, Czechia

Abstract

The two stork species that nest in Central Europe, *Ciconia ciconia* and *Ciconia nigra*, have been repeatedly shown to host the digenetic trematode *Cathaemasia hians* (Rudolphi, 1809) in their esophagus and muscular stomach. These host species differ in their habitat and food preferences, and the morphologic characters of *C. hians* isolates ex *Ci. nigra* and *Ci. ciconia* are not identical. These differences led to a previous proposal of two subspecies, *Cathaemasia hians longivitellata* Macko, 1960, and *Cathaemasia hians hians* Macko, 1960. We hypothesize that the *Cathaemasia hians* isolates ex *Ci. nigra* and *Ci. ciconia* represent two independent species. Therefore, in the present study, we performed the first molecular analyses of *C. hians* individuals that were consistent with the diagnosis of *C. hians hians* (ex *Ci. nigra*) and *C. hians longivitellata* (ex *Ci. ciconia*). The combined molecular and comparative morphological analyses of the central European *Cathaemasia* individuals ex *Ci. nigra* and *Ci. ciconia* led to the proposal of a split of *C. hians* into *C. hians* sensu stricto (formerly *C. hians hians*) and *C. longivitellata* sp. n. (formerly *C. hians longivitellata*). Morphological analyses confirmed that the length of the vitellaria is the key identification feature of the two previously mentioned species. Both *Cathaemasia* spp. substantially differ at the molecular level and have strict host specificity, which might be related to differences in the habitat and food preferences of the two stork species.

Introduction

The digenetic trematode *Cathaemasia hians* (Rudolphi, 1809) was initially described from the black stork *Ciconia nigra*. Later, *C. hians* has been repeatedly shown to be hosted by both stork species that nest in Central Europe, *Ciconia ciconia* and *Ci. nigra* (*C. nigra*: Viborg 1795; Rudolphi 1809, 1819; Nathusius 1837; Dujardin 1845; von Willemoes Suhm 1873; Müller 1897; Mühling 1898; Yoshida and Toyoda 1930; Szidat 1940a; Macko 1960b; Van den Broek 1963; Gundlach 1969; Merino et al. 2001; Saad 2009; Liptovszky et al. 2012; Hampl and Sitko 2013; Königová et al. 2015; Sitko and Heneberg 2015; Ramilo et al. 2021; *C. ciconia*: Gurlt 1845; Baird 1853; van Beneden 1868; Mühling 1897; Van den Broek 1960, 1963; Mettrick 1963; Grünberg and Kutzer 1964; Gundlach 1969; Schuster et al. 2002; Sitko and Heneberg 2015; Michalczyk et al. 2020; Sitko and Heneberg 2021). These stork species differ substantially in diet. The black stork *Ci. nigra* feeds predominantly on fish and, to a lesser extent, on amphibians and mollusks and hunts for them in wetlands, particularly slow-flowing waters (Merino et al. 2001; Liptovszky et al. 2012). In contrast, Czech populations of the white stork *Ci. ciconia* feed mainly on mammals and earthworms, with amphibians present in the diet in the past but only rarely in recent years (Reif et al. 2006; Voříšek 2006); fish are absent from the common prey types of this bird species. The dominant diet types may differ with respect to the landscape context and can be seasonal. The main feeding habitats of *Ci. ciconia* include arable fields, dry pastures, and rubbish dumps (Alonso et al. 1991; Carrascal et al. 1993). The diet change in Czech populations of *Ci. ciconia* was hypothesized to be associated with the recent decline in *C. hians* prevalence in *Ci. ciconia* of Czech origin (Sitko and Heneberg 2021). Although the findings of *C. hians* from other host species are known, only a recent report of *C. hians* from *Aquila heliaca* (Aves: Accipitridae) (Juhásová et al. 2023) represents a correctly identified specimen, whereas the findings of *C. hians* in *Ardea cinerea*, *Ardea purpurea*, and *Nycticorax nycticorax* (all Aves: Ardeidae) by Parona (1899) represented misidentifications (The only individuals from *A. heliaca* were not fixed on slides but lysed for DNA isolation. The first author of the cited study, Ľ. Juhásová, refused to share the deposited DNA or to analyze the species identity of this individual (Ľ. Juhásová, in litt.); thus, the species identity of this finding remains unclear.) Other possible satellite hosts include *Ardea*

cinerea (Stossich 1891), *Ardea goliath* (Dollfus 1950), and *Hydrocoloeus minutus* (Condorelli-Francaviglia 1897).

We hypothesized that the *Cathaemasia hians* isolates ex *Ci. nigra* and *Ci. ciconia* represent two independent species. Therefore, in the present study, we performed the first molecular analyses of *C. hians* individuals that were consistent with the diagnosis of *C. hians hians* (ex *Ci. nigra*; Table 2) and *C. hians longivitellata* (ex *Ci. ciconia*; Table 3), providing integrative evidence to support the reclassification of *C. hians longivitellata* as a standalone species.

Materials and methods

Sampling

We examined helminths from carcasses of storks provided dead for deposition in the Comenius Museum in Přerov. The birds died from various causes at various sites in the Czech Republic (48° 39′N–50°59′N, 12°19′E–18°29′E), Central Europe. We examined the carcasses immediately or froze them and examined them within two months of receipt. For the phylogenetic analyses, we fixed representative individuals of helminths in 96% ethanol from May 2011 to May 2022 for further analyses. A complete list of the sequenced individuals is provided in Table 1. For the comparative morphological analyses, we stained another set of 45 *C. hians* sensu lato individuals (30 ex *Ci. nigra* and 15 ex *Ci. ciconia*) in Semichon's carmine, followed by dehydration through an alcohol series, and we then mounted the helminths in Canada balsam. For the analyses of egg length, we measured the longest egg present within each examined adult individual. The body measurements are shown as the range (mean ± standard deviation) and are presented in μm unless otherwise specified.

We also measured the material deposited on slides by Macko (1960a) and provide measurements of the holotype and seven paratypes diagnosed by Macko as *C. hians longivitellata* (ex *Ci. ciconia*) and eight adult individuals diagnosed by Macko as *C. hians hians* (ex *Ci. nigra*). We included only individuals with eggs in these measurements. These materials are currently deposited at the Institute of Parasitology, Czech Academy of Sciences in České Budějovice, Czech Republic.

DNA extraction, amplification, and sequencing

We extracted, amplified, and sequenced the DNA using primers that targeted nuclear ribosomal DNA (partial 18S rDNA and ITS2) and mitochondrial (CO1 and ND1) loci as described by Heneberg et al. (2018). We submitted the resulting visually checked sequences to NCBI GenBank under accession numbers OR533419 (18S rDNA), OR533496-OR533499 (ITS2), OR536618-OR536623 (CO1), OR544075 (ND1), PP157883-PP157886 (ND1), PP177534-PP177536 (18S rDNA), and PP177542-PP177543 (ITS2) (Table 1).

Alignments and phylogenetic analyses

We aligned the obtained sequences and publicly available sequences of closely related species retrieved from NCBI GenBank as of February 8, 2024, along with sequences of corresponding outgroups (selected as sequences of species with the highest similarity of the sequence of the respective locus to the sequences of the same locus obtained from *Cathaemasia* spp. and publicly available in NCBI GenBank at a time when the analyses were performed) using ClustalW (with the following parameters: gap opening penalty 7 and gap extension penalty 2 for both pairwise and multiple alignments, DNA weight matrix IUB, and transition weight 0.1). We manually corrected any inconsistencies in alignments and trimmed the alignments to the length of the shortest sequence. The trimmed 18S rDNA locus (partial SSU rRNA coding sequence) corresponded to nt. 63-1741 (1679 bp) of *Petasiger phalacrocoracis* (Echinostomatidae) AY245709.1. The trimmed ITS2 locus (partial 5.8S ribosomal RNA, full-length ITS2, and partial 28S ribosomal RNA sequences) corresponded to nt. 2478-3196 (719 bp) of *Isthmiophora hortensis* (Echinostomatidae) AB189982.1. The trimmed CO1 locus (partial CO1 coding sequence) corresponded to nt. 7626-7955 (330 bp) of *Fasciolopsis buski* (Fasciolidae) NC_030528.1. The trimmed ND1 locus (partial ND1 coding sequence) corresponded to nt. 13-365 (352 bp) of *Echinochasmus coaxatus* (Echinochasmidae) MN720147.1.

We calculated the maximum likelihood fits of the 24 nucleotide substitution models for each locus. We employed a bootstrap procedure with 1,000 replicates and nearest-neighbor interchange as the maximum likelihood heuristic method of choice for tree inference when we generated the initial tree using a neighbor-joining algorithm. We then determined the best substitution model based on the lowest Bayesian Information Criterion scores and used best-fit models for the maximum likelihood phylogenetic analyses. The models used to construct the maximum likelihood phylogenetic trees were the Kimura 2-parameter model with gamma-distributed rates among sites (18S rDNA and ITS2) and the Hasegawa-Kishino-Yano model with gamma-distributed rates among sites (five discrete gamma categories) (CO1 and ND1). We also used these models to estimate the evolutionary divergence between sequences. We conducted all the maximum likelihood analyses in MEGA5.

To validate the maximum likelihood analysis data, we employed Bayesian inference. We converted the ClustalW alignments generated in MEGA5 to the Nexus format in Mesquite 3.04. We then

Table 1. New sequences of *Cathaemasia* spp. that were collected from Czechia and generated throughout the course of the present study (NCBI GenBank accession numbers are indicated)

Specimen	Species, host, age/sex, sampling site, country, sampling date	Locus 18S rDNA	ITS2	CO1	ND1
3LF–2331	*Cathaemasia hians* ex *Ciconia nigra*, 1Y, Hrabyně, Czechia, 30-Aug–2011	PP177534	OR533496	OR536618	
3Lf–2332	*Cathaemasia hians* ex *Ciconia nigra*, M, Huslenky, Czechia, 23-May–2011	PP177535	OR533497	OR536619	PP157883
3LF–2333	*Cathaemasia hians* ex *Ciconia nigra*, 1Y, Komorní Lhotka, Czechia, 23-Jul–2011	PP177536	OR533498	OR536620	PP157884
3LF–3989	*Cathaemasia hians* ex *Ciconia nigra*, 1Y, Bartošovice, Czechia, Jun–2016	OR533419	OR533499	OR536621	PP157885
3LF–4426	*Cathaemasia longivitellata* sp. n. ex *Ciconia ciconia*, M, Bartošovice, Czechia, 10-May–2022		PP177542	OR536622	PP157886
3LF–4427	*Cathaemasia longivitellata* sp. n. ex *Ciconia ciconia*, M, Bartošovice, Czechia, 10-May–2022		PP177543	OR536623	OR544075

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Table 2. Measurements of *Cathaemasia hians* sensu stricto based on adult individuals ex *Ciconia nigra* (data are shown as a range [mean ± standard deviation]; measurements are shown in μm)

Measure	Present study (n = 30)	Macko (1960a) [dimensions in square brackets are valid for living individuals][1]	Fixed individuals deposited by Macko (1960a) (n = 8)[2]	Braun (1901) (identified as *C. fodicans* ex *Chlidonias niger*)[3]	Yoshida and Tomoda (1930)	Ramilo et al. (2021) (n = 10)[4]	Saad (2009)	Königová et al. (2015) (n = 9)
Body length	7,436–16,159 (10,986)	[5,600–14,200]	9,721–13,638 (11,771)	7,500	9,000–13,850 (10,560)	5,760–7,200 (6,500)	9,450–11,700	9,940 ± 1,700
Body width	2,814–5,710 (3,801)	2,140–5,200	3,947–5,005 (4,396)	2,500	3,420–4,690 (3,820)	2,780–3,860 (3,240)	2,070–3,780	3,480 ± 880
Body length/width ratio	1: 2.14–3.56 (3.02)		1: 2.08–3:46 (2.70)					
Body width [% of body length]	28%–47%		28.9%–47.9% (38.2%)					
Forebody	2,414–3,140 (2,776)		3,140–4,662 (3,845)					
Hindbody	4,420–5,430 (5,058)		5,205–8,180 (6,750)					
Forebody/hindbody ratio	1: 1.52–2.24		1: 1.30–2.00 (1.68)					
Forebody [% of body length]	27%–34%		29.3%–36.1% (32.8%)					
Number of spines	12–19 (17)							
Oral sucker	542–1,143 × 626–1,143 (749 × 816)	654–973 × 654–973 [746–1,018 × 746–1,018]	714–857 × 714–1,000 (761 × 875)	633 × 700	640–930 × 400–840 (730 × 510)	684–895 (809)	450–630 × 540–810	760 ± 120
Ventral sucker	904–1,514 × 940–1,486 (1,109 × 1,145)	651–990 × 651–990 [913–1,357 × 913–1,357]	943–1,286 × 943–1,314 (1,122 × 1,154)	1,000 × 1,000	970–1,590 × 940–1,530 (1,170 × 1,130)	916–1548 (1184)	900–1,134	1,120 ± 400
Sucker length ratio	1: 1.32–1.67 (1.36)		1: 1.22–1.80 (1.48)	1: 1.58				
Sucker width ratio	1: 1.3–1.5 (1.4)		1: 1.06–1.84 (1.34)	1: 1.43				
Prepharynx	312–415 (410)	[422]	86–343 (207)				144–270	
Pharynx	422–602 × 443–771 (527 × 541)	422–588 × 422–588 [497–701 × 497–701]	343–743 × 514–760 (564 × 634)	500 × 333	450–650 × 490–650 (580 × 520)		414–450 × 450–656	
Esophagus length	216–714 (440)		596–1,000 (774)					
Anterior testes	714–2,220 × 1,200–3,718 (1,314 × 1,877)	[1,244–2,602 × 1,244–2,602]	1,000–1,571 × 1,743–2,029 (1,262 × 1,925)				810–900 × 864–1,008	
Posterior testes	572–2,571 × 586–3,146 (1,277 × 1,466)	[1,267–2,330 × 1,267–2,330]	1,029–1,714 × 1,457–2,000 (1,503 × 1,646)				860–990 × 810–900	
Post-testicular space / body length ratio	5%–15%		7.3%–14.6% (11.6%)					

(*Continued*)

Table 2. (*Continued*)

Measure	Present study (n = 30)	Macko (1960a) [dimensions in square brackets are valid for living individuals][1]	Fixed individuals deposited by Macko (1960a) (n = 8)[2]	Braun (1901) (identified as *C. fodicans* ex *Chlidonias niger*)[3]	Yoshida and Tomoda (1930)	Ramilo et al. (2021) (n = 10)[4]	Saad (2009)	Königová et al. (2015) (n = 9)
Post-testicular space length/body length ratio	17%–30%		20.5%–26.4% (23.6%)					
Cirrus pouch	482–1,429 × 361–1,429 (762 × 635)	[701–1,289 × 520–973]	600–886 × 429–914 (607 × 636)		620–1,050 × 400–750 (760 × 490)		245–560 × 140–245	
Ovary	216–571 × 180–514 (293 × 333)		200–629 × 314–486 (322 × 364)		230–370 × 140–250 (280 × 180)		320–325 × 231–240	
Mehlis gland	241–361 × 265–578 (321 × 422)		286–629 × 343–714 (375 × 464)					
Left vitellarium branch	2,860–5,710 (4,423)		6,149–8,723 (7,451)					
Right vitellarium branch	2,823–5,600 (4,205)		6,850–8,408 (7,621)					
Vitellarium/body length ratio	48%–64% (55%)		59.8%–68.7% (63.2%)					
Uterus length	2,571–6,061							
Uterus/body length ratio	33%–43%		35.8%–47.2% (40.3%)					
Egg	97–108 × 54–62 (105 × 59)	118–124 × 45–51	87–103 × 54–62 (98 × 60)	72–83 × 42	71–83 × 41–55		90–98 × 51–55	

[1]Some of the measurements provided by Macko (1960a) are valid for individuals that were alive at the time of the measurement; these measurements are shown in square brackets.
[2]We double-checked and measured the material deposited on slides by Macko (1960a) and provide measurements of the eight individuals identified by Macko (1960a) as *C. hians hians* (including only individuals with eggs). The differences between measurements of live (published by Macko 1960a) and pressure-fixed individuals (materials collected by Macko 1960a measured in the present study) are caused by pressure fixation and by the application of a series of 96% ethanol, carboxylol, and xylol in the course of the fixation of the latter individuals.
[3]The data from Braun (1901) were reported originally for *Cathaemasia fodicans* ex *Sterna nigra* (the host identity was not confirmed by Braun himself, but it was retrieved from the Vienna Museum label; Braun only measured the archived specimen). Later authors, including Odhner (1926), Yoshida and Toyoda (1930), and Szidat (1939), suggested that the examined specimen represented *C. hians* and that the label of the host species was probably erroneous and should be *Ciconia nigra.*
[4]The measurements provided by Ramilo et al. (2021) likely contained an erroneously positioned decimal point in measurements of oral and ventral suckers, which were claimed to be one order of magnitude larger than commonly observed values; the data provided in this table include the correction of this obvious error.

Table 3. Measurements of *Cathaemasia longivitellata* sensu Macko (1960a) based on adult individuals ex *Ciconia ciconia* (data are shown as a range [mean ± standard deviation]; measurements are shown in μm)

Measure	Present study (n = 15)	Macko (1960a) [dimensions in square brackets are valid for living individuals][1]	Fixed holotype individual deposited by Macko (1960a)[2]	Variability of fixed type individuals deposited by Macko (1960a) (n = 7)	Iskova (1985)	Feizullaev (1961)	Mettrick (1963) (immature individual)
Body length	10,000–15,710 (12,189)	5,900–16,000	15,500	10,725–16,588 (14,590)	6,300–7,000	10,500–21,000	10,400
Body width	3,430–5,290 (4,396)	2,090–5,700	6,290	3,710–6,290 (5,123)	3,200–3,500	3,500–7,000	2,900
Body length/width ratio	1: 2.33–3.58 (2.79)		1: 2.64	1: 2.41–3.37 (2.89)			
Body width [% of body length]	42%–53%		40.6%	29.6%–41.5% (35.0%)			
Forebody	2,657–4,860 (3,693)	2,890–4,183	4,147	3,430–5,140 (4,229)	2,600–2,750		
Hindbody	6,290–9,140 (7,346)		10,010	6,290–10,010 (9,061)			
Forebody/hindbody ratio	1: 1.72–2.50		1: 2.41	1: 1.69–2.58			
Forebody [% of body length]	29%–36%		26.7%	25.6%–33.6% (29.1%)			
Number of spines	20–36 (31)						
Oral sucker	685–1,120 × 629–1,143 (847 × 916)	613–1,131 × 654–1,040	914 × 857	771–1,086 × 771–1,143 (898 × 943)	640–700 × 700–820	732–1,080 × 876–1,380	640 × 700
Ventral sucker	971–1,486 × 1,000–1,486 (1,244 × 1,210)	613–1,131 × 613–1,131 [1,357 × 1,357]	1,343 × 1,343	1,143–1,514 × 1,171–1,629 (1,355 × 1,376)	1,100–1,200 × 1,200–1,300	1,120–1,620 × 1,150–1,680	1,160 × 1,120
Suckers` length ratio	1: 0.53–1.67 (1.42)		1: 1.47	1: 1.39–1.73 (1.52)			
Suckers` width ratio	1: 1.12–2.01 (1.36)		1: 1.57	1: 1.30–1.58 (1.47)			
Prepharynx	57–200 (141)	[422 × 422]	57	57–371 (164)	550–780	120–240	
Pharynx	400–596 × 429–714 (490 × 530)	422–588 × 313–452 [656 × 565]	687 × 514	514–687 × 486–600 (575 × 551)	480–520 × 450–500	540–660 × 554–792	440 × 460
Esophagus length	600–1,143 (890)		771	771–1,514 (1,110)			640
Anterior testes	1,143–3,000 × 1,426–3,430 (1,682 × 2,077)	[1,856–3,172 × 1,856–3,172]	2,114 × 3,140	1,571–2,860 × 2,086–3.575 (2,037 × 2,694)	1,300–1,980 × 1,650–2,820	1,260–2,400 × 1,920–4,440	
Posterior testes	1,120–2,143 × 1,286–2,428 (1,420 × 1,907)	[1,762–2,820 × 1,762–2,820]	2,571 × 2,600	1,286–2,571 × 1,657–2,289 (2,049 × 2,290)		1,260–3,120 × 1,720–4,020	
Post-testicular space/body length ratio	7%–12%		3.0%	3.0%–9.7% (6.6%)			
Testes length (combined)/body length ratio	19%–36%		31.0%	24.0%–31.7% (27.1%)			
Cirrus sac	522–1,371 × 536–1,371 (763 × 845)	[728–1,242 × 634–1,195]	114 × 857	886–1,514 × 714–2,000 (988 × 1,172)	580–950 × 650–700	780–1,596 × 780–1,680	800 × 270
Cirrus	2,143 × 143						
Ovary	429–571 × 457–514 (500 × 486)	[320–470 × 329–822]	457 × 571	286–543 × 328–743 (431 × 590)	350–400 × 480–500	360–660 × 420–840	240 × 280
Mehlis gland	134–429 × 209–457 (338 ×344)		343 × 541	286–629 × 286–600 (355 × 494)			

(*Continued*)

Table 3. (*Continued*)

Measure	Present study (n = 15)	Macko (1960a) [dimensions in square brackets are valid for living individuals][1]	Fixed holotype individual deposited by Macko (1960a)[2]	Variability of fixed type individuals deposited by Macko (1960a) (n = 7)	Iskova (1985)	Feizullaev (1961)	Mettrick (1963) (immature individual)
Left vitellarium branch	7,430–12,860 (9,839)		12,440	9,009–12,727 (11,326)			
Right vitellarium branch	8,000–12,860 (10,181)		11,783	8,290–12,870 (10,588)			
Vitellarium/body length ratio	68%–97% (85%)		80.3% and 76.0%	71.0%–84.0% (78.8%)			
Uterus length	4,860–6,543						
Uterus/body length ratio	38%–48% (40%)		38.0%	37.1%–46.7% (42.3%)			
Egg	87–104 × 54–62 (99 × 59)	105–112 × 45–51	93 × 54	87–104 × 54–62 (99 × 60)	95–110 × 52–55	72–102 × 24–42	74–81 × 34–47

[1]Some of the measurements provided by Macko (1960a) are valid for individuals that were alive at the time of the measurement; these measurements are shown in square brackets.
[2]We double-checked and measured the material deposited on slides by Macko (1960a) and provide measurements of the holotype and six paratypes (including only individuals with eggs). The differences between measurements of live (published by Macko 1960a) and pressure-fixed individuals (materials collected by Macko 1960a measured in the present study) are caused by pressure fixation and by the application of a series of 96% ethanol, carboxylol, and xylol in the course of the fixation of the latter individuals.

performed the Bayesian analysis using the mixed model of nucleotide substitution in MrBayes 3.2.5. We used four Monte Carlo Markov chains for 10,000,000 generations and trees sampled every 1,000th generation, with the average standard deviation of split frequencies not exceeding 0.0030. We discarded the first 25% of samples as burn-in. We used the remaining dataset to generate a 50% majority-consensus tree with the posterior probabilities of branches indicated and visualized the resulting trees in FigTree 1.4.2. We obtained the following summary statistics for analyses performed: average standard deviation of split frequencies 0.006–0.025, maximum standard deviation of split frequencies 0.017–0.090, average potential scale reduction factor 1.000–1.005, and maximum potential scale reduction factor 1.000–1.007.

Results

Molecular phylogenetics

We sequenced and analyzed differences between *C. hians hians* (ex *Ci. nigra*) and *C. hians longivitellata* (ex *Ci. ciconia*) using three DNA loci representing hypervariable DNA regions (CO1, ND1, and ITS2). We also sequenced partial 18S rDNA, but we were able to amplify this locus for only one of the subspecies. Molecular phylogenetic analyses of the three hypervariable regions provided clear support for the elevation of *C. hians longivitellata* (ex *Ci. ciconia*) to the species level (Fig. 1). Of particular interest were the analyses of CO1 (Fig. 1A) and ITS2 (Fig. 1E), which illustrate well the genetic distance between the two proposed species. The conclusions from maximum likelihood analyses were confirmed using the Bayesian approach (Fig. S1).

The genetic distance between the CO1 loci of *C. hians hians* (ex *Ci. nigra*) and *C. hians longivitellata* (ex *Ci. ciconia*) was 12.8%. There was no intraspecific genetic variability among the sequences of the respective *Cathaemasia* spp. The genetic distance between the ITS2 loci of *C. hians hians* (ex *Ci. nigra*) and *C. hians longivitellata* (ex *Ci. ciconia*) was 4.2%-4.5%. The intraspecific variability among *C. hians hians* (ex *Ci. nigra*) was 0.0% to 0.1%, and the variability was 0.3% among isolates of *C. hians longivitellata* (ex *Ci. ciconia*).

Species descriptions

***Cathaemasia longivitellata* Macko sp. n.**

Synonym: *Cathaemasia hians longivitellata* Macko, 1960

Host: *Ciconia ciconia* (Aves: Ciconiiformes) (prevalence 4.1 %; intensity of infection 1–14 individuals).

Location in host: Esophagus, muscular stomach.

Locality: Czech Republic: Strachotín (48.90°N, 16.65°E).

Other localities: Czech Republic: Bartošovice (49.66°N, 18.05° E), Záhlinice (49.29°N, 17.48°E).

Examined specimens: Type specimen and six paratype specimens D534/2, all collected by J. K. Macko; currently in the collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic. Additional 15 specimens P-P-1865/1, all in the collection of Comenius Museum, Přerov, Czech Republic. All represent adult individuals with eggs present. DNA samples are deposited at the Charles University, Third Faculty of Medicine, Prague, Czech Republic (marked as 3LF-4426 and 3LF-4427).

Zoobank accession: The Life Science Identifier for *Cathaemasia longivitellata* sp. n. is urn:lsid:zoobank.org:act:D6DCBD4B-B1FA-4600-84BE-A1C1DC5A2CC5.

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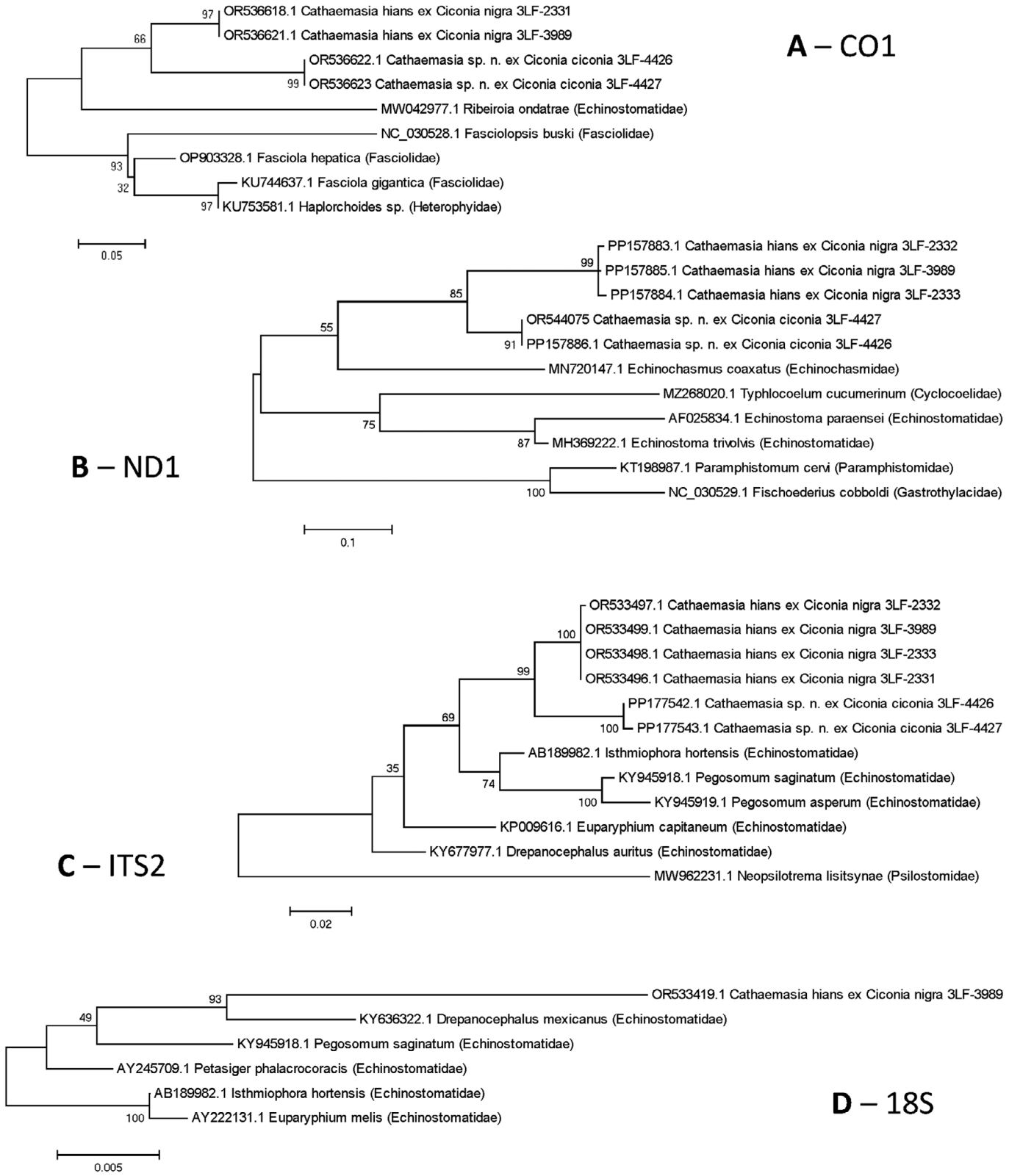

Figure 1. Maximum likelihood analyses of the sequences of the mitochondrial and nuclear DNA loci of *Cathaemasia* spp. (A) CO1, (B) ND1, (C) ITS2, and (D) 18S rDNA. The bars indicate the number of substitutions per nucleotide. The numbers above the internodes indicate the percentage of trees in which the associated taxa clustered together.

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DNA sequences: ITS2: PP177542 and PP177543; CO1: OR536622 and OR536623; ND1: PP157886 and OR544075.

Etymology: The specific epithet longivitellata is identical to the name previously proposed by Macko (1960a), who was the first to recognize the morphologic distinctness of this species and propose its status as a subspecies *Cathaemasia hians longivitellata.* The species name refers to a characteristic identification feature of this species: the prominent length of vitellaria relative to the total body length.

Description (15 specimens ex *Ciconia ciconia*) (Fig. 2A-B): Body medium to large, elongately oval, with rounded extremities and maximum width at mid hindbody, 10,000–15,710 × 3,430–5,290 (12,189 × 4,396), body length/width ratio 1: 2.33–3.58 (2.79) (body width equals to 42%–53 % of body length). Forebody length 2,657–4,860 (3,693), hindbody length 6,290–9,140 (7,346), forebody/hindbody length ratio 1: 1.72–2.5 (2); forebody occupies 29% to 36 % of the body length. Post-testicular field length 714 to 1,571; post-testicular field occupies 7% to 12% of body length. Tegument wrinkled, thickness 220 to 480, greater in median portions than lateral margins. Dorsal and ventral thicknesses of tegument similar. Cercariae have collar with 47 spines (Bykhovskaya-Pavlovskaya and Kulakova 1977); in adult digeneas collar rudimentary, with only 20 to 36 (31) small, sharply pointed spines, 24 to 70 × 13–27, in two lateral groups. Body covered with scales, approximately one third smaller in juveniles than adults. Scales in area of suckers round shaped, on anterior body edge larger than on lateral body edges, median edge of oral sucker 34 × 34, lateral margin 24 × 24, scales of ventral sucker median edge 48 × 48, and lateral margin 48 × 42. Scales oval-shaped on remaining parts of body: scales from esophagus and intestinal bifurcation 45 × 42, scales of ovary and testes medially 64 × 71, and lateral margin 54 × 64, behind posterior testes 62 × 62. Oral sucker spherical, small 685 to 1,120 × 629 to 1,143 (847 × 916). Ventral sucker spherical, longer than oral sucker, in second quarter of body 971 to 1,486 × 1,000 to 1,486 (1,244 × 1,210), oral/ventral suckers length ratio 1: 0.53 to 1.67 (1.42), width ratio 1: 1.12 to 2.01 (1.36). Prepharynx short, 57 to

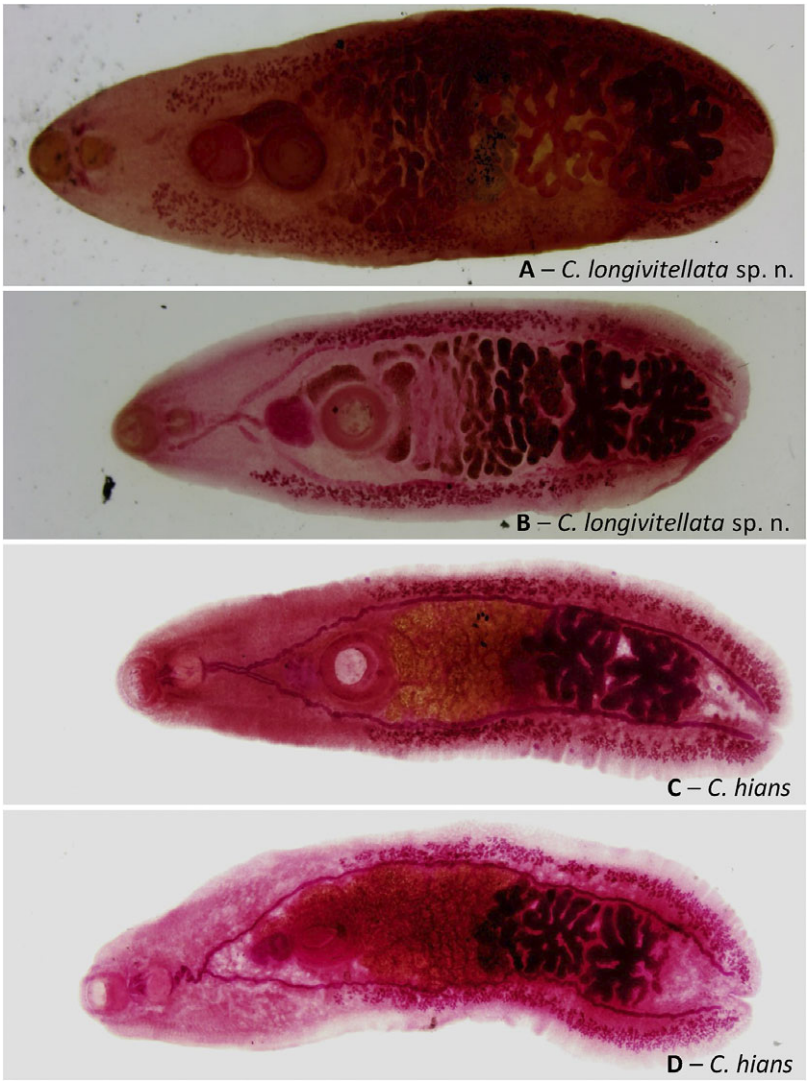

Figure 2. Representative photographs of *C. longivitellata* sp. n. (A, B) and *C. hians* sensu stricto (C, D). (A) *Cathaemasia longivitellata* sp. n. ex *Ciconia ciconia*, female, May 1, 1967, Napajedla, district Zlín, Czech Republic, site: esophagus. (B) *Cathaemasia longivitellata* sp. n. ex *Ciconia ciconia*, male, July 27, 1999, Nošovice, district Frýdek-Místek, Czech Republic, site: esophagus. (C-D) *Cathaemasia hians* ex *Ciconia nigra*, female, May 28, 1976, Šišma, district Přerov, Czech Republic, site: esophagus.

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200 (141). Pharynx globular, 400 to 596 × 429 to 714 (490 × 530). Esophagus moderately long, 600 to 1.143 (890), without lateral diverticula. Intestinal bifurcation approximately halfway between pharynx and ventral sucker. Ceca sinuous, with short outer lateral diverticula. Testes large, contiguous, deeply lobed to branched, in posterior quarter of body. Anterior testis 1,143 to 3,000 × 1,426 to 3,430 (1,682 × 2,077), always slightly larger than posterior 1,120 to 2,143 × 1,286 to 2,428 (1,420 × 1,907). Cirrus pouch elongately oval, entirely anterior to ventral sucker 522 to 1,371 × 536 to 1,371 (763 × 845). Internal seminal vesicle large, saccate. Prostatic pars short. Genital pore median, approximately halfway between intestinal bifurcation and ventral sucker. Ovary small, elongate or round, submedian, postequatorial, 429 to 571 × 457 to 514 (500 × 486). Mehlis´ gland diffuse, contiguous with ovary, 134 to 429 × 209 to 457 (338 × 344). Vitellarium in two compact lateral small follicles, from pharynx or halfway between pharynx and ventral sucker up to posterior body extremity, left branch 7,430 to 12,860 (9,839), right branch 8,000 to 12,860 (10,181). Vitellarium occupies 68% to 98% (85%) of body length. Stem of excretory vesicle may bear lateral diverticula, pore terminal. Uterus long 4,860 to 6,543 (uterus occupies 38%–48% [40%] of body length), loops numerous between ovary and ventral sucker, may overlap ceca. Metraterm indistinct. Eggs numerous, relatively small 87 to 104 × 54 to 62 (99 × 59), contain fully developed miracidium with distinct eyespots.

Remarks: Specialized parasite of white storks (*Ciconia ciconia*) in Europe and Africa. The two newly proposed *Cathaemasia* spp. differ mainly in the length of their vitellaria. However, as the total body length is highly variable in both of these species, we propose using the vitellaria length ratio to the total body length. In *C. hians* sensu stricto, the vitellaria/body length ratio is 48% to 64% (57%), whereas it is 67% to 97% (82%) of the total body length in *C. longivitellata* sp. n. The genetic distance between the CO1 loci of *C. hians* sensu stricto and *C. longivitellata* sp. n. was 0.128 base substitutions per site. An example of characteristic *C. longiviellata* sp. n. sequence is GGGTTTGGATGTTC (CO1 locus; position 153–166 in OR536622), whereas the sequence of this locus in *C. hians* sensu stricto is AGGTTTAGATGTAC.

Note: Pressure-fixed holotype and six paratypes collected by Macko (1960a) were re-examined; the measurements are provided in Table 3, and photograph of the holotype is provided in Fig. 3A. Some of the individuals deposited by Macko were subadults and did not have developed eggs. We measured only those with eggs, which caused differences in the lower ranges of some of the measurements.

***Cathaemasia hians* (Rudolphi, 1809) Looss, 1899**

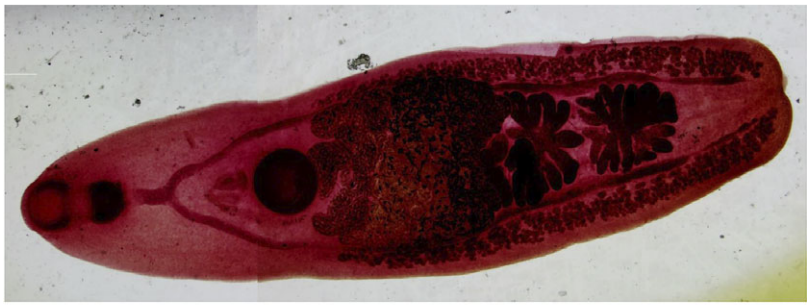
Synonym: *Distoma hians* Rudolphi, 1809; *Cathaemasia hians hians* Macko, 1960

Host: *Ciconia nigra* (Aves: Ciconiiformes) (prevalence 41.2 %; intensity of infection 1–32 individuals).

Location in host: Esophagus, muscular stomach.

Localities: Czech Republic: Bartošovice (49.66°N, 18.05°E), Hrabyně (49.87°N, 18.03°E), Huslenky (49.29°N, 18.09°E), Komorní Lhotka (49.66°N, 18.49°E), Přerov (49.45°N, 17.46°E), Záhlinice (49.29°N, 17.48°E).

A – *C. longivitellata* sp. n. holotype, leg. J. K. Macko



B – *C. hians* sensu stricto leg. J. K. Macko

Figure 3. Photographs of the *C. longivitellata* sp. n. holotype (A, ex *Ciconia ciconia*, 1957, Senné, Slovakia, site: esophagus) and representative individual of *C. hians* sensu stricto (B, ex *Ciconia nigra*, undisclosed date, Košický region, Slovakia, site: esophagus), both collected and prepared by Macko (1960a). Photographs were merged from two images each. Specimens in the collection by J. K. Macko were not numbered individually, only the holotype was labeled.

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Examined specimens: Specimens D534/1, all collected by J. K. Macko; currently in the collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic. Additional 30 specimens P-P-1865/1, all in the collection of the Comenius Museum, Přerov, Czech Republic. All represent adult or subadult individuals with eggs present. DNA samples were deposited at Charles University, Third Faculty of Medicine, Prague, Czech Republic (marked as 3LF-2331, 3LF-2332, 3LF-2333, and 3LF-3989).

DNA sequences: 18S rDNA: PP177534-6, OR533419; ITS2: OR533496-9; CO1: OR536618-21; ND1: PP157883-5.

Description (30 specimens ex *Ciconia nigra*) (Fig. 2C-D): Digenea pink to freshly red in color. Body medium to large, elongately oval, with rounded extremities and maximum width at mid-hindbody, 7,436 to 16,159 × 2,814 to 5,710 (10,986 × 3,801), body length/width ratio 1: 2.14 to 3.56 (3.02) (body width equals to 28%–47% of body length). Forebody length 2,414 to 3,140 (2,776), hindbody length 4,420 to 5,430 (5,058), forebody/hindbody length ratio 1: 1.52 to 2.24 (1.84); forebody occupies 27% to 34% of the body length. Post-testicular field length 571 to 1,286; post-testicular field occupies 6% to 15% of body length. Tegument wrinkled, thickness 220 to 480, greater in median portions than lateral margins. Dorsal and ventral thickness of tegument similar. Cercariae likely have collars with 47 spines (Bykhovskaya-Pavlovskaya and Kulakova 1977), in adult digeneas collar rudimentary, with only 24 to 36 (34) small, sharply pointed spines 40 to 75 × 19 to 27 in two lateral groups. Body covered with scales, approximately one third smaller in juveniles than in adults. Scales in area of suckers round shaped, on anterior edge larger than on lateral edges, median edge of oral sucker 31 × 26, lateral margin 16 × 26, scales of ventral sucker: median edge 48 × 48, and lateral margin 42 × 48. Scales oval-shaped on remaining parts of the body: scales from esophagus and intestinal bifurcation 32 × 54, scales of ovary and testes medially 48 to 51 × 64 to 71, lateral margin 48 × 48, behind posterior testes 64 × 64. Oral sucker subglobular 542 to 1,143 × 626 to 1,143 (749 × 816). Ventral sucker spherical, longer than oral sucker, in second quarter of body, 904 to 1,514 × 940 to 1,486 (1,109 × 1,145). Oral/ventral suckers length ratio 1: 1.32 to 1.67 (1.36), width ratio 1: 1.3 to 1.5 (1.4). Prepharynx short, 312 to 415 (410). Pharynx long oval, 422 to 602 × 443 to 771 (527 × 541). Esophagus short, 216 to 714 (440), without lateral diverticula. Intestinal bifurcation approximately halfway between pharynx and ventral sucker. Ceca sinuous, with short outer lateral diverticula, long, reaches up to middle distance of sucker. Testes large, contiguous, deeply lobed to branched, in posterior quarter of body. Anterior testis 714 to 2,571 × 1,200 to 3,718 (1,374 × 1,877), always slightly larger than posterior 572 to 2,220 × 686 to 3,146 (1,277 × 1,466). Cirrus pouch elongate oval, entirely anterior to ventral sucker 482 to 1,429 × 361 to 1,429 (762 × 635). Internal seminal vesicle large, saccate. Prostatic pars short. Cirrus tubular, unarmed. Genital pore median, approximately halfway between intestinal bifurcation and ventral sucker. Ovary small transversely elongate or round, submedian, postequatorial 216 to 571 × 180 to 514 (293 × 333). Mehlis´ gland diffuse, contiguous with ovary 241 to 361 × 265 to 578 (321 × 422). Vitellarium nonconfluent, in two compact laterals extra cecal fields of small follicles composed of individual follicles, reach from rear edge of ventral sucker up to posterior body extremity, left branch 2,860 to 5,710 (4,423) and right branch 2,823 to 5,600 (4,205). Vitellarium occupies 48% to 64% (55%) of body length. Stem of excretory vesicle may bear lateral diverticula, pore terminal. Uterus long 2,571 to 6,061 (uterus occupies 33%–43% of body length), loops numerous, between ovary and ventral sucker, may overlap ceca. Metraterm indistinct. Eggs numerous, relatively small 97 to 108 × 54 to 62 (105 × 59), contain fully developed miracidium with distinct eyespots.

Remarks: Specialized parasite of black storks (*Ciconia nigra*) in Europe and Africa.

Note: The largest individuals of both species (*C. hians* and *C. longivitellata*, sp. n.) were of similar size. Eight pressure-fixed individuals collected by Macko (1960a) were re-examined; the measurements are provided in Table 2, and a photograph of the representative slide is provided in Fig. 3B. Some of the individuals deposited by Macko were subadults and did not have developed eggs. We measured only those with eggs, which caused differences in the lower ranges of some of the measurements.

Discussion

Several previous studies have noted morphological differences between the proposed species. First, Macko (1960a) proposed the existence of two subspecies, *C. hians hians* and *C. hians longivitellata*. A year later, Feizullaev (1961) described a new species, *Cathaemasia skrjabini* ex *Ciconia ciconia* from Azerbaijan. Feizullaev noted that the vitellaria of *C. skrjabini* extend anteriorly to the level of the genital bursa. The same author also proposed an alternative explanation in his follow-up study (Feizullaev 1962), claiming that the reported morphological differences might result from development in different intermediate hosts. That Feizullaev (1961) described the new species ex *Ci. ciconia* based on material from the Transcaucasian region (Azerbaijan) caused a somewhat chaotic situation when some Western European parasitologists, such as Van den Broek (1963), continued to recognize the materials from European *Ci. nigra* and *Ci. ciconia* as *C. hians* but accepted the materials from Azerbaijani *Ci. ciconia* as *C. skrjabini* sensu Feizullaev (1961). Other authors, including Gundlach (1969), recognized both subspecies, confirming their strict host specificity.

Another issue associated with descriptions of *C. hians* by previous authors stems from the absence of mentions of host species in some of the descriptions or from the mixing of data from both host species. For example, Szidat (1940b) published a drawing of *C. longivitellata* sp. n. ex *Ci. ciconia*. However, the text of his study does not recognize *C. longivitellata* sp. n. and proposes that previously suggested *C. fodicans* (here synonymized with *C. longivitellata* sp. n.) ex *Chlidonias niger* is identical to *C. hians* and that the host published by Braun (1901) was in fact *Ci. nigra* (which is most likely an accurate claim). Later, Chiriac and Udrescu (1973) reprinted the *C. longivitellata* sp. n. drawing from Szidat (1940b) but claimed that it was hosted by *Ci. nigra*.

Several researchers provided measurements that were identical to those published by other authors earlier but mentioned themselves as the authors of the measurements. This applies, for example, to the descriptions of *C. hians* from Hungary by Edelényi (1974), who provided identical measurements as Lühe (1909). However, the descriptions by Lühe (1909) were also not original because they were identical to those provided by Braun (1902). Surprisingly, this does not correspond to the provided illustrative drawings because Edelényi (1974) published a drawing of *C. longivitellata* sp. n., whereas Lühe (1909) published a drawing of *C. hians*. Additionally, Bykhovskaya-Pavlovskaya and Kulakova (1977) provided *C. hians* measurements, but these were identical to those published by Macko (1960a), who remained uncited by Bykhovskaya-Pavlovskaya and Kulakova (1977).

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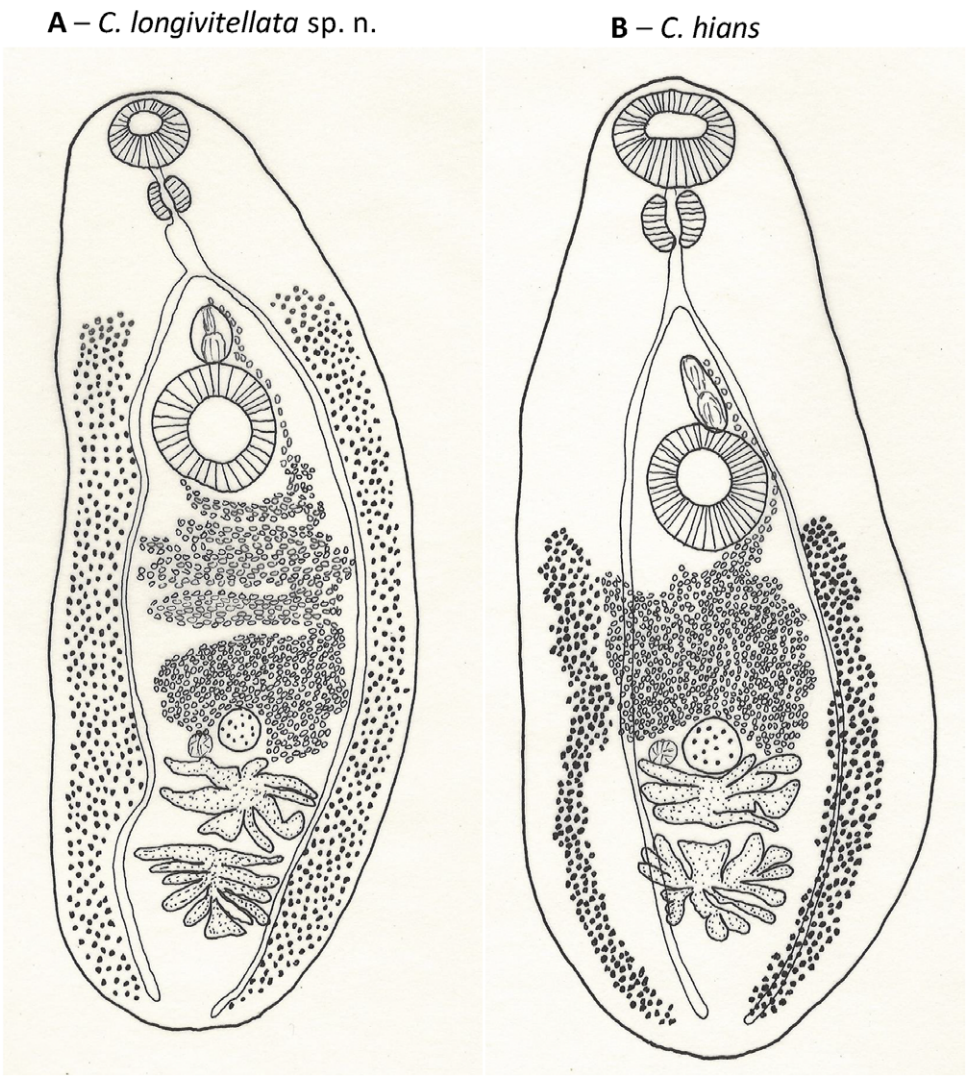

Figure 4. Drawings of *C. longivitellata* sp. n. (A) and *C. hians* sensu stricto (B).

Some previous host-parasite records were erroneous. *Ardea cinerea*, *Ardea purpurea*, and *Nycticorax nycticorax* were reported as *C. hians* hosts in Italy by Parona (1899), but these records represented erroneously identified trematodes of the genus *Clinostomum.*

In addition to the above-named species, the *C. hians* species complex also contains the African species *Cathaemasia variabilis.* This species was described from *Sphenorhynchus abdimii* in Africa and was recognized as valid by Van den Broek (1963) but was synonymized with *C. hians* as *C. hians variabilis* by Bykhovskaya-Pavlovskaya and Kulakova (1977). The extent of vitellaria is identical to that of *C. hians.* Therefore, without subsequent DNA analyses, it is impossible to draw conclusions regarding the systematics of members of the *C. hians* complex in tropical and subtropical regions outside Europe.

In our view, valid descriptions of individuals of *C. hians* sensu stricto were published by Macko (1960a), Yoshida and Tomoda (1930), and Braun (1901). We provide a comparative table of measurements provided by these authors in Table 2. Note that the drawing and description in Braun (1901) is consistent with the *C. hians* sensu stricto diagnosis. Nevertheless, the author claimed that the host was *Chlidonias niger* (identified as *Sterna niger* according to the taxonomy valid at a time of the description). However, it is unlikely that the black tern was infected by the species strictly specialized to *Ci. nigra*, and we assume that the host was recorded erroneously. The valid descriptions of individuals of *C. longivitellata* sp. n. were provided by Macko (1960a), Feizullaev (1961), and Iskova (1985), and these descriptions are compared in Table 3.

The intensity of infection by *C. hians* sensu stricto was greater than that by *C. longivitellata* sp. n., which contributes to the apparently smaller size of individuals of this species. The largest individuals of both *Cathaemasia* spp. are of similar size. Because of the dietary changes of both examined stork species, particularly from the nearly complete dietary change of the Czech population of *C. ciconia* from amphibians to small mammals and other types of dietary items (Sitko and Heneberg 2021), both *Cathaemasia* spp. have become rare in recent years. These species were dominant among the trematodes of both stork species, but recently, we had to examine more than 100 stork individuals to find them only once in the past 10 years.

Species identity of *C. hians* sensu lato findings from intermediate hosts remains to be elucidated. The first intermediate hosts of

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C. hians sensu lato are snails; the repeatedly reported hosts are *Planorbis planorbis* (Baršienė 1991; Zhytova and Korol 2012; Tkach et al. 2016) and *Lymnaea stagnalis* (Grabda-Kazubska et al. 1990; Baršienė 1990; Faltýnková et al. 2008). The presence of *C. hians* sensu lato in the first of these two species was also confirmed by molecular analysis (Tkach et al. 2016). Other Planorbiidae and Lymnaeidae are also hypothesized to be permissive intermediate hosts (Szidat 1939; Zhytova & Korol 2012); for example, infections of *Planorbis* and *Anisus* spp. were reported by Zdun (1961). Notably, the karyotypes of *C. hians* sensu lato isolated from *P. planorbis* and *L. stagnalis* differed from one another (Baršienė 1991). It is unclear whether these isolates of *C. hians* sensu lato represented different species or which of the isolates should be assigned to *C. hians* sensu stricto. The second intermediate hosts of *C. hians* sensu lato are amphibian tadpoles, including those of *Bombina bombina*, *Pelophylax ridibundus*, *Pelophylax esculentus*, and Ranidae spp. (Volgar-Pastukhova 1959; Vojtková 1982; Grabda-Kazubska and Lewin 1989). It is unclear whether other vertebrate species may also serve as second intermediate hosts. Merino et al. (2001) proposed that *C. hians* sensu lato requires a warm climate to complete its life cycle. However, the authors mentioned above provided multiple pieces of evidence of the presence of infected snails and amphibians locally (Poland, Czech Republic, Lithuania, and Ukraine – Sandner 1949; Zdun 1961; Vojtková and Křivanec 1970; Balúsek and Vojtek 1973; Baršienė 1990, 1991; Grabda-Kazubska et al. 1998; Faltýnková et al. 2008; Zhytova and Korol 2012). Vojtková (1982) examined 1536 amphibian tadpoles from 82 sampling sites across the Czech Republic and Slovakia, reporting differences in *C. hians* sensu lato prevalence from zero up to 35% (*Pelophylax esculentus* tadpoles from Palkovičovo (recently termed Sap), Slovakia). Studies of adult amphibians often conclude the absence of *C. hians* sensu lato metacercariae in adult frogs (Kozák 1973) at localities where highly prevalent *C. hians* sensu lato infections of storks are known (Macko 1961). Supporting the completion of the life cycle locally, there is also evidence of infection of juvenile *C. nigra*, which was approximately 76 days old, in the Czech Republic (Hampl and Sitko 2013), and infection of *C. nigra* nestlings in Spain was reported by Merino et al. (2001). The second author of the present study also found two flightless nestlings at sampling sites Strachotín and Napajedla (both Czech Republic), which were positive for *C. hians* sensu lato (J. Sitko, pers. obs.).

In conclusion, combined molecular and comparative morphological analyses of central European *Cathaemasia* individuals ex *Ci. nigra* and *Ci. ciconia* led to the proposal of a split of *C. hians* into *C. hians* sensu stricto (formerly *C. hians hians* sensu Macko 1960a) and *C. longivitellata* sp. n. (formerly *C. hians longivitellata* sensu Macko 1960a). Morphological analyses confirmed that the length of the vitellaria was the key identification feature of the two above-mentioned species. Both *Cathaemasia* spp. have strict host specificity, which might be related to differences in food preferences of the two stork species, and they substantially differ at the molecular level.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000622.

Declaration.

Ethical approval. Not applicable. All the host birds were obtained dead and therefore no ethics permit was required by Czech law. The research on bird helminths was authorized by the Ministry of the Environment of the Czech Republic; the most recent permit was issued on August 3, 2009, under No. 11171/ENV/09-747/620/09-ZS 25.

Availability of data and materials. Representative specimens of the helminths analysed in this study are available in the collections of the Comenius Museum in Přerov. All data are available in the main text or the supplementary materials.

Competing interest. On behalf of both authors, the corresponding author states that there is no conflict of interest.

Author contribution. P.H. performed the molecular and phylogenetic analyses and wrote the manuscript; J.S. conceived the study, examined the host birds, and performed the morphological analyses. Both authors revised the manuscript and agreed on its final version.

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