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Hemolivia and *Hepatozoon*: Haemogregarines with Tangled Evolutionary Relationships

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The generic name *Hemolivia* has been used for haemogregarines characterized by morphological and biological features. The few molecular studies, focused on other haemogregarine genera but involving *Hemolivia* samples, indicated its close relationship to the genus *Hepatozoon*. Here we analyze molecular data for *Hemolivia* from a broad geographic area and host spectrum and provide detailed

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morphological documentation of the included samples. Based on molecular analyses in context of other haemogregarines, we demonstrate that several sequences deposited in GenBank from isolates described as *Hepatozoon* belong to the *Hemolivia* cluster. This illustrates the overall difficulty with recognizing *Hemolivia* and *Hepatozoon* without sufficient morphological and molecular information. The close proximity of both genera is also reflected in uncertainty about their precise phylogeny when using 18S rDNA. They cluster with almost identical likelihood either as two sister taxa or as monophyletic *Hemolivia* within paraphyletic *Hepatozoon*. However, regardless of these difficulties, the results presented here provide a reliable background for the unequivocal placement of new samples into the *Hemolivia*/*Hepatozoon* complex.

Key words: Apicomplexa; haemogregarines; *Hemolivia*; *Hepatozoon*; host specificity; phylogeny.

Introduction

Apicomplexa, as an extremely rich and diversified group of protozoan parasites, have traditionally been considered a phylogenetically and evolutionarily challenging taxon. Hypotheses explaining some of the profound questions about their deep origin have been perceived as important landmarks in the research of this group (e.g. position of cryptosporidia; Carreno et al. 1999). However, apart from such fundamental questions, the apicomplexan phylogeny still contains many uncertainties regarding the origin and phylogenetic status of various more recent crown groups. Previous phylogenetic analyses within these groups revealed unexpected relationships conflicting with morphology and/or biology (e.g. *Cyclospora* within *Eimeria*, or *Isospora* vs. *Cystoisospora*; Barta et al. 2005; Pieniazek and Herwaldt 1997; Relman et al. 1996). The genus *Hemolivia* is a representative of such crown groups within apicomplexans with fairly unresolved phylogenetic relationships and evolutionary history. This genus comprises tick-transmitted haemogregarines of ectothermic vertebrates, with only three described species so far; *H. stellata* (the type species) from the Neotropic cane toad *Rhinella marina* (formerly *Bufo marinus*), *H. mariae* from the Australian sleepy lizard *Tiliqua rugosa*, and *H. mauritanica* from the Palaearctic spur-thighed tortoise *Testudo graeca* (Petit et al. 1990; Sergent and Sergent 1904; Smallridge and Paperna 1997). *Hemolivia* differs from the closely related genus *Hepatozoon*, by several characteristics of its biology. First, sporogony is divided into two phases; in the first phase, oocysts with sporokinetes are formed, whereas the formation of sporocysts and sporozoites takes place during the second phase. On the contrary, the genus *Hepatozoon* is characterized by large polysporocystic oocysts. Intraerythrocytic merogony was described in *Hemolivia* (Petit et al. 1990), whereas it has never been observed in *Hepatozoon* (Desser 1993). *Hemolivia* gamonts occurring in the peripheral blood of vertebrate hosts are typical for the presence of a stain-resistant parasitophorous vacuole. These gamonts are morphologically recognizable by their cylindrical or slightly elliptical shape with a straight long axis, differing from the frequently curved structures of related genera, e.g. *Hepatozoon* and *Haemogregarina*. The mature gamonts of *Hemolivia* resemble empty sticks with a blue-stained nucleus at the polar position.

While morphologically and biologically well-defined, *Hemolivia* has been largely neglected in terms of phylogenetic analyses and its position within the haemogregarines remains unclear. The only two molecular studies, which also include a *Hemolivia* species, are a brief study dealing with the molecular detection of *Hemolivia* in ticks from *Testudo graeca* from Algeria (Harris et al. 2013), and a comprehensive analysis focused on the phylogeny of adeleorinids, mainly of the genus *Hepatozoon* (Barta et al. 2012). While the latter analysis is informative with respect to the phylogeny of *Hepatozoon*, it remains uncertain regarding the position of *Hemolivia*. In the phylogenetic tree presented by Barta et al. (2012), *Hemolivia mariae* clusters within *Hepatozoon* as a sister taxon of a sample labeled as *Hepatozoon* sp. (GenBank acc. no. EU430236) from the brown water python *Liasis fuscus*. This arrangement poses serious questions on the monophyly and phylogenetic position of the genus *Hemolivia*. The short distance between *Hemolivia* and the closest *Hepatozoon* indicates a relatively recent split between these two lineages. This implies that *Hemolivia*, with its current diversity and distribution, is either a surprisingly recent group or is a non-monophyletic assemblage of morphologically defined organisms.

There are precedents in the apicomplexan taxonomy of seemingly monophyletic groups, defined morphologically and biologically that turned out to

be polyphyletic morphotypes after examination with molecular markers. *Eimeria maxima*, a coccidium infecting poultry, serves as an example (Schwarz et al. 2009), and we also detected similar phenomenon when analyzing eimerians from rodents (Kvičerová et al., unpubl. observ.). However, *Hemolivia* monophyly can certainly not be ruled out based on the position of a single sample. Moreover, Barta et al. (2012) admit that the closest relative to *H. mariae* in their tree may have been determined incorrectly and could represent another *Hemolivia* rather than *Hepatozoon*.

So, there are currently several possible scenarios for the phylogeny and evolution of *Hemolivia*, including 1) polyphyly of *Hemolivia*, 2) *Hepatozoon* and *Hemolivia* as two monophyletic sister groups, or 3) paraphyly of *Hepatozoon* with respect to a monophyletic *Hemolivia*. Here, we present molecular data for a geographically and taxonomically representative set of *Hemolivia*, analyze them in the context of other haemogregarines and discuss possible evolutionary and taxonomical implications.

Results

Prevalence and Morphology of Blood Stages

During microscopic examination of blood smears we detected parasites corresponding morphologically to the two described *Hemolivia* species, namely *H. mauritanica* in 326 *Testudo graeca* and 41 *T. marginata* (Supplementary Material Fig. S1a-d), and *H. mariae* in 6 *Egernia stokesii* (Supplementary Material Fig. S1e-h). For detailed morphological descriptions see Supplementary Material Text S2. Since in vertebrate hosts, premerogonic stages and meronts occur in peripheral blood particularly at the beginning of infection and soon afterwards they change into gamonts (Široký et al. 2007), this latter stage markedly prevailed in the field-collected blood smears. The samples from 10 *R. pulcherrima manni* were infected by haemogregarines of distinct hemolivian morphology, not corresponding to any so far described species of *Hemolivia*. This *Hemolivia* is described as follows.

Hemolivia sp. ex *Rhinoclemmys pulcherrima manni* (Fig. 1a-d)

Description of the morphology of blood stages: The single early merogonic stage detected measured $10.0 \times 6.0\ \mu m$. Its shape was ovoid, possessing a vacuolated and heterogeneous cytoplasm with scattered chromatin. Merozoites were rarely observed in the bloodstream, being elongated and much slimmer than meronts, and measuring $8.0 \times 4.0\ \mu m$ (n = 3). The cytoplasm was vacuolated and the nucleus was not clearly visible. Gamonts were found in erythrocytes as single individuals, exceptionally in pairs. They were slightly rounded and elongated, localized in a stain-resistant capsula. Mature gamonts resembled empty bars with a thin blue smear representing the nucleus located at the polar position. The thin blue line along the long axis of the capsule indicated the curved position of the gamont inside the capsule. The capsule measured $8.2 \pm 0.8 \times 4.9 \pm 0.5\ \mu m$ (7.0 – 10.0 × 4.0 – 6.0; n = 63), with LW $40.6 \pm 6.37\ \mu m^2$ (28 - 60) and L/W ratio 1.68 ± 0.19 (1.3 - 2.25). The nuclei measured $3.1 \pm 0.6 \times 1.6 \pm 0.6\ \mu m$ (2.0 - 4.0 × 1.0 - 3.0) with LW $5.0 \pm 2.14\ \mu m^2$ (2.0 - 12.0).

Description of oocysts, sporocysts and sporozoites: Not found, the definitive host is unknown.

Host*: Rhinoclemmys pulcherrima manni* (Dunn, 1930) (Testudines: Geoemydidae).

Other hosts: Unknown.

Locality: South Nicaragua, exact locality unknown.

Distribution: So far known only from the single locality.

Prevalence: 10 of 30 *R. pulcherrima manni* were positive (33.3 %).

Site of infection: Blood samples collected from live turtles, parasites detected in peripheral blood.

Material: Blood films (marked as NIC-4-13 and NIC-10-13) and DNA samples no. 5054 and 5060 are deposited in collection of Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

Molecular features: 18S rDNA sequence of 1421 bp (GenBank accession number KF992714), GC content 39.4%.

Remarks: Gamonts of *Hemolivia* isolates from *R. pulcherrima manni* are on average shorter (8.2 μm) than gamonts of *H. mauritanica* (13.3 μm), *H. stellata* (9 μm), and *H. mariae* (8.9 μm), their shape is more rounded and the nucleus is much smaller. Nevertheless, the widths of gamonts of all *Hemolivia* species are very similar and their structure is rather uniform and does not allow a reliable differential diagnosis based only on the gamont's morphology (Lainson et al. 2007; Petit et al. 1990). Since no molecular data are available for *H. stellata*, the *Hemolivia* samples from *R. pulcherrima manni* cannot be distinguished with certainty from

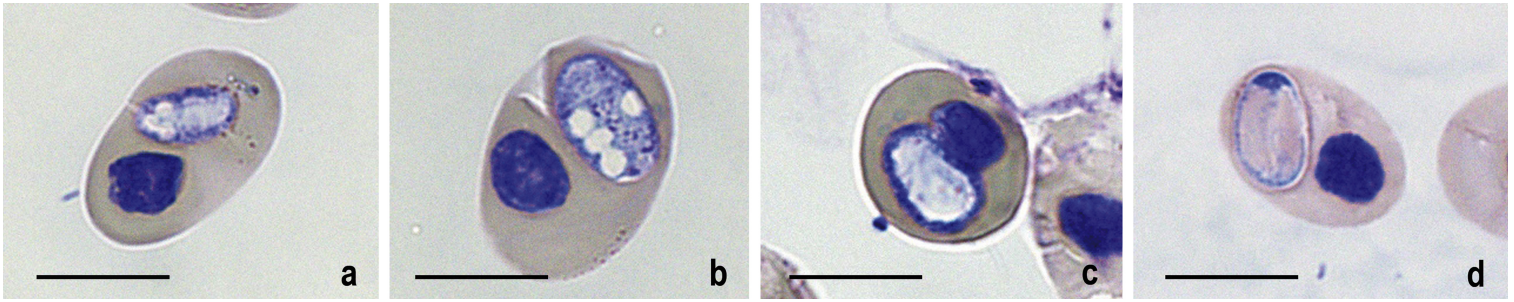

Figure 1. Photomicrographs of endogenous stages of the *Hemolivia* species in the peripheral blood of the turtle *Rhinoclemmys pulcherrima manni*, all in the same scale; scale bar = 10 μm.
a) supposed transition form between merozoite and early merogonic stage; **b**) early merogonic stage; **c**) young gamont; **d**) mature gamont.

H. stellata, that is also distributed in the neotropical zoogeographic region.

Molecular Characteristics and Phylogeny

Of the positive blood samples, partial 18S rDNA sequences were obtained for 32 samples of *H. mauritanica*, 4 samples of *H. mariae*, and 10 samples of *Hemolivia* from *R. pulcherrima manni*. Details on sequences and their accession numbers are provided in the Supplementary Material Table S3. The lengths and informative content of the alignments varied with the composition of individual matrices (Table 1).

All phylogenetic analyses agreed on a robust monophyletic *Hemolivia-Hepatozoon* cluster composed of three main well-supported monophyletic lineages: 1) *Hemolivia*, 2) host-specific *Hepatozoon* from carnivores, and 3) *Hepatozoon* from other host taxa. The *Basic matrix* produced a topology with *Hemolivia* clustering within *Hepatozoon*, making the latter genus paraphyletic (Fig. 2; Table 1). However, several modifications of the matrix showed that this is not the only possible arrangement supported by the data. Random selection of the taxa included into the analysis lead occasionally to alternative topology with *Hepatozoon* and *Hemolivia* forming two monophyletic sister taxa (Supplementary Material Fig. S4). A comparison of these two possible relationships between *Hepatozoon* and *Hemolivia* by the likelihood ratio (SH test) shows convincingly that neither of them is significantly preferred by the data ($p = 0.39$). An extension of the *Basic matrix* with additional samples (including short or poorly overlapping sequences) further complicated the relation between the phylogeny and host specificity. More specifically, the samples isolated from lacertid lizards clustered together with the carnivore-specific *Hepatozoon*s rather than within the broad-host-range cluster (Fig. 3, Supplementary Material Fig. S5).

Discussion

Monophyly and Diversity of *Hemolivia*

Hemolivia, as a distinct genus of haemogregarines, was originally defined based on morphological and biological features (Petit et al. 1990) (the typical morphology of *Hemolivia* gamonts for the samples included in this study is documented in Fig. 1 and Supplementary Material S1-S2). However, number of examples including several apicomplexan groups show that from a phylogenetic perspective, such definition may be misleading (Barta et al. 2005; Pieniazek and Herwaldt 1997; Relman et al. 1996). Therefore, to investigate phylogenetic status and relationships of the haemogregarines corresponding to the *Hemolivia* morphology, we built a data set covering a broad geographic area and host spectrum. In the trees inferred from the *Basic matrix*, all these *Hemolivia* samples clustered as a well-supported monophyletic group firmly nested within haemogregarines, with *Hepatozoon* as a close relative. Although an exact relationship between *Hemolivia* and *Hepatozoon* remains uncertain and apparently not accessible with the current data (i.e. the 18S rDNA), the phylogenetic arrangement of *Hemolivia* and the *Hepatozoon* lineages has several evolutionary and taxonomical implications. Most importantly, since the data set representing the genus *Hemolivia* contains samples from a broad range of hosts and geographical locations (Table 2, Supplementary Material Table S3 and Fig. S6), the monophyly of these morphologically determined samples is likely to reflect a single common origin for these parasites, rather than an artifact of improper sampling, and therefore supports the phylogenetic status of the genus *Hemolivia* as a monophyletic group.

This may be of importance when dealing with previously sequenced samples of unknown morphology, or with any samples for which only DNA is available. For example, the sample of the GenBank acc. no. EU430236 designated as *Hepatozoon* in the study of Barta et al. (2012) clustered firmly within *Hemolivia* in our trees, suggesting that this sample was misidentified *Hemolivia* rather than *Hepatozoon*. Consequently, this change of the phylogenetic status of the sequence no. EU430236 moves the split between the *Hemolivia* and *Hepatozoon* clusters deeper into the haemogregarine phylogeny. Another biologically interesting example is provided by the phylogenetic study of Maia et al. (2012). Their analysis, focused on the phylogeny of *Hepatozoon*, presents a polytomy composed of four presumable *Hepatozoon* lineages. One of the lineages only contains three samples: two of them isolated from *Amblyomma* ticks (collected on the monitor lizard *Varanus panoptes* and the brown water python *Liasis fuscus*), and the third isolated from the mosquito *Aedes taeniorhynchus*. When introduced into our data set (the *Extended matrix)*, these samples were scattered across the *Hemolivia* cluster (Fig. 3). It is obvious that in Maia et al. (2012), these three samples were misidentified as *Hepatozoon* due to the absence of any *Hemolivia* in their matrix. This view is further supported by the fact that the two samples with known hosts

Table 1. Information on phylogenetic analyses of molecular data and parameters used.

Matrix	BI (MrBayes)	ML (Phyml)	MP (PAUP)
Basic 51 sequences, alignment length 904 bp outgroup: *Babesiosoma*, *Dactylosoma*, haemogregarines	GTR + Γ + I mcmc = 10,000,000 gens. burn-in = 25%	GTR + Γ + I 1000 replicates -lnL: 4107.187257	hsearch TBR 1000 replicates best tree = 489, 50% majority-rule consensus of 64 trees CI = 0,6789
	Hemolivia + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C	*Hemolivia* in polytomy
Long 65 sequences, alignment length 1362 bp outgroup: *Babesiosoma*, *Dactylosoma*, haemogregarines	GTR + Γ + I mcmc = 10,000,000 gens. burn-in = 25%	GTR + Γ + I 1000 replicates -lnL: 5975.509981	hsearch TBR 1000 replicates best tree = 677, 50% majority-rule consensus of 100 trees CI = 0,6706
	Hemolivia + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C
Alternative 59 sequences, alignment length 1515 bp outgroup: *Babesia*, *Eimeria*, *Goussia*, *Theileria*	GTR + Γ + I mcmc = 10,000,000 gens. burn-in = 25%	GTR + Γ + I 1000 replicates -lnL: 9794.180449	hsearch TBR 1000 replicates best tree = 1495, 50% majority-rule consensus of 100 trees CI = 0,6094
	Hemolivia + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C
Extended 81 sequences, alignment length 1526 bp outgroup: *Babesiosoma*, *Dactylosoma*, haemogregarines	GTR + Γ + I mcmc = 10,000,000 gens. burn-in = 25%	GTR + Γ + I 1000 replicates -lnL: 7259.450347	hsearch TBR 1000 replicates best tree = 833, 50% majority-rule consensus of 100 trees CI = 0,6351
	Hemolivia + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C	*Hemolivia* + *Hepatozoon* C
Monophyletic 57 sequences, alignment length 904 bp outgroup: adelines, *Klossia*	GTR + Γ + I mcmc = 10,000,000 gens. burn-in = 25%	GTR + Γ + I -lnL: 5050.495062	hsearch TBR 1000 replicates best tree = 682, 50% majority-rule consensus of 100 trees CI = 0,6569
	Hepatozoon monophyletic	*Hepatozoon* monophyletic	*Hemolivia* + *Hepatozoon* C

Hepatozoon A – clade of *Hepatozoon* spp. infecting carnivores
Hepatozoon B – clade of *Hepatozoon* spp. infecting lacertid lizards
Hepatozoon C – clade of *Hepatozoon* spp. infecting various groups of hosts (marsupials, rodents, reptiles, amphibians)

Table 2. List of all analysed sequences of *Hemolivia* spp. and the groups of identical sequences.

Sequence group (haplotype) no.	Isolate no.	Locality	Host
Hemolivia mauritanica			
1	TR-8-08	Turkey, Muş	*Testudo graeca*
2	SY-49-05	Syria, Ayn Dara	
3	SY-10-10-2	Syria, Qalaat Semaan	
4	SY-10-10-3	Syria, Qalaat Semaan	
5	SY-28-10-1	Syria, Tele Karamah	
6	SY-28-10-2	Syria, Tele Karamah	
7	SY-28-10-3	Syria, Tele Karamah	
8	SY-45-10	Syria, Ayn Dara	
9	SY-72-10	Syria, Krak des Chevaliers	
10	SY-20-10-3	Syria, Qalaat Semaan	
11	IQ-4-10	Iraq, Sulajmanija Region	
	IQ-1-10	Iraq, Sulajmanija Region	
	TR-21-08	Turkey, Hakkâri	
	TR-25-08	Turkey, Şemdinli	
	TR-26-08	Turkey, Şemdinli	
	TR-30-08	Turkey, Van	
	sp. 1 DJH-2013	Algeria	
	SY-80-05	Syria, Qalaat Semaan	
	SY-36-07	Syria, Qalaat Semaan	
	SY-41-07	Syria, Cyrrhus	
	SY-48-07	Syria, Kafr Takharim	
	SY-8-10	Syria, Qalaat Semaan	
	SY-12-10	Syria, Qalaat Semaan	
	SY-15-10	Syria, Qalaat Semaan	
	SY-67-10	Syria, Krak des Chevaliers	
	SY-68-10	Syria, Krak des Chevaliers	
	IR-15-11	Iran, Siah Dar-e	
	IR-20-11	Iran, Sultanabad	
	IR-29-11	Iran, Sultanabad	
	IR-32-11	Iran, Ourumyieh-Sera	
	GR-16-04	Greece, Volos	*Testudo marginata*
12	GR-9-04	Greece, Volos	
13	Vendelin	Greece, Platamonas	
Hemolivia mariae			
14	4903	South Australia, Hawker, Flinders Ranges	*Egernia stokesii*
	4954	South Australia, Hawker, Flinders Ranges	
	4956	South Australia, Hawker, Flinders Ranges	
15	4955	South Australia, Hawker, Flinders Ranges	
16	mariae JN211118	South Australia, Mt. Mary	*Tiliqua rugosa*
Hemolivia* sp. ex *Rhinoclemmys pulcherrima manni			
17	5060	southern Nicaragua	*Rhinoclemmys pulcherrima manni*
	5056	southern Nicaragua	
	5062	southern Nicaragua	
	5067	southern Nicaragua	
	5077	southern Nicaragua	
	5079	southern Nicaragua	
18	5054	southern Nicaragua	
	5052	southern Nicaragua	
	5065	southern Nicaragua	
	5069	southern Nicaragua	

(i.e. *Varanus panoptes* and *Liasis fuscus*) from Australia cluster in our tree together with Australian isolates from lizards *Egernia stokesii* and *Tiliqua rugosa* (Fig. 3). These two examples demonstrate the importance of a reliable phylogenetic framework for the taxonomical placement and biological interpretation of such closely related taxa. Considering the host and geographic range of the included *Hemolivia* samples and their considerable genetic variation, we believe that the presented data set provides such framework and will allow, in most cases, for placing of any new sample reliably within one of these two genera.

Phylogenetic Relationships between *Hemolivia* and *Hepatozoon*

While the monophyly of *Hemolivia* (i.e. the samples of "hemolivian morphology") is convincingly established, the phylogenetic relationship between *Hemolivia* and *Hepatozoon* is much less clear. Two competing arrangements have been published for this phylogenetic problem, one places *Hemolivia* within *Hepatozoon* making the latter genus paraphyletic (Barta et al. 2012), the other dealing with *Hemolivia* and *Hepatozoon* as two monophyletic sister taxa (Harris et al. 2013). We addressed this uncertainty via two additional analyses. When inferring trees from randomly selected taxon data sets, we found that the mutual position of these lineages varied with the matrix composition (an example of *Hepatozoon*-monophyly solution is shown in Supplementary Material Fig. S4). Further, the SH tests performed on the two different topologies, i.e. 1) monophyletic *Hepatozoon* vs. 2) *Hemolivia* inserted within paraphyletic *Hepatozoon*, revealed not only a lack of a significant difference, but the two likelihood values were almost identical (-ln ML 4266 and 4262, respectively; $p = 0.39$). Under current conditions, i.e. with the molecular data available, it is not possible to decide on the exact position of *Hemolivia* in respect to *Hepatozoon*. Considering the difficulties with obtaining appropriate samples and informative sequences, this situation may not be easily remedied in the near future.

Phylogenetic Significance of Host Specificity and Geography

In *Hemolivia*, host specificity to poikilotherm intermediate hosts and ticks as vectors/definitive hosts has been previously established for the described species (Petit et al. 1990; Sergent and Sergent 1904; Smallridge and Bull 1999; Smallridge and Paperna 1997). In our *Hemolivia* samples from *R. pulcherrima manni*, the definitive host is unknown, but the biology of the terrestrially-living turtle host suggests that this role may be also played by ticks. It is therefore interesting to note that one of the samples clustering with *Hemolivia* samples (Maia et al. 2012) has been isolated from mosquito, indicating that ticks may not be exclusive vectors of this genus. However, such evidence should be treated with caution as the detection of the parasite in a blood-feeding arthropod (either by molecular tools or microscopy) does not necessarily confirm a functional vector-parasite association. Regarding intermediate hosts, the situation is even less clear. Each of the two previously described species, *H. mauritanica* and *H. mariae*, seems to be specific to different "reptile" groups, Testudines and Squamata, respectively (Godfrey et al. 2006; Široký et al. 2004, 2009; Smallridge and Paperna 1997). Inclusion of the third species, *Hemolivia* from *R. pulcherrima manni* thus makes the Testudine-specificity paraphyletic. Moreover, the type species of the genus *Hemolivia* – *H. stellata*, has been proven to infect two evolutionary distant intermediate hosts – the cane toad *Rhinella marina* and the giant ameiva lizard *Ameiva ameiva* (Lainson et al. 2007). It is clear, however, that the current taxon sampling is too limited to attempt any reliable analysis of the evolution of the host specificity of *Hemolivia*.

Regarding the phylogenetic background, the only *Hemolivia* species sufficiently sampled for a reasonable evaluation of the geographic distribution within phylogenetic framework is *H. mauritanica*. This monophyletic cluster covers a large geographic area without any phylogenetic-geographic pattern. Rather, the sequences from the whole distribution form a largely unresolved polytomy, suggesting relatively high gene flow across the area. The best illustration of this phenomenon is provided by the haplotype 11, representing 21 samples that cover the whole distribution area from the easternmost to the westernmost borders. This intriguing finding indicates that it will certainly be interesting to further investigate the means of *Hemolivia* dispersion regarding its host specificity and biology.

Methods

Sampling: *Hemolivia* samples were obtained from three chelonian and one lizard species. Altogether, 594 *Testudo graeca* and 47 *T. marginata* tortoises were sampled in the Mediterranean region by walking through habitat during the years 2001-2012.

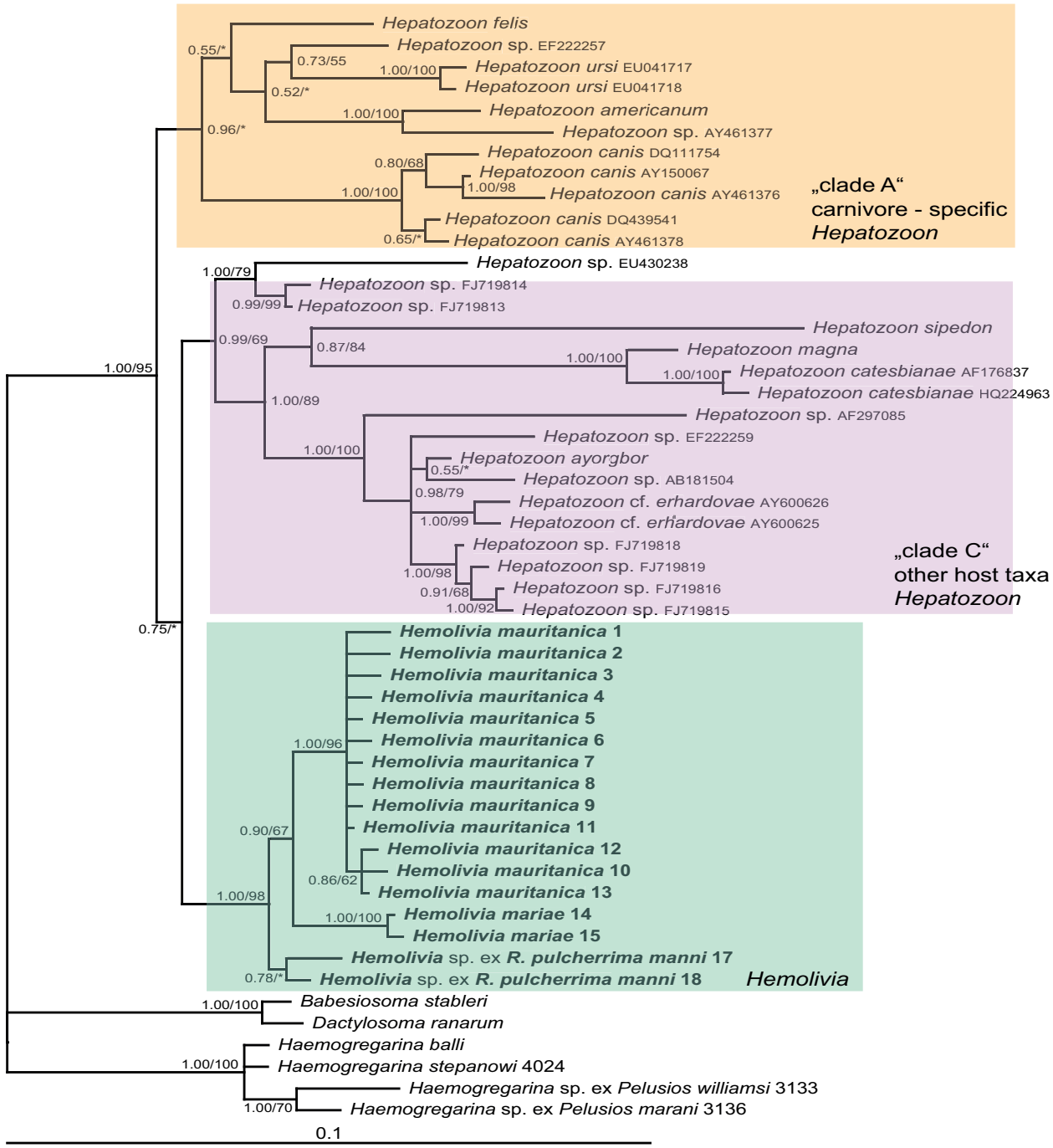

Figure 2. BI and ML tree derived from *Basic matrix*. Numbers at the nodes show posterior probabilities under BI / bootstrap values for ML. Posterior probabilities and bootstrap supports lower than 0.50 or 50 %, respectively, are marked with asterisk (*).

The tortoises were released at the same place immediately after the sampling procedures. Thirty pet-traded *Rhinoclemmys pulcherrima manni* originating in Nicaragua were sampled in March 2013 during veterinary screening in the Czech Republic. Six *Egernia stokesii* lizards were sampled during September and October 2012 in the southern Flinders Ranges near Hawker, South Australia. Blood for microscopic and molecular analyses was collected by puncture of the caudal vein. Blood smears were air-dried, then fixed in absolute methanol for 5 min, and stained with Giemsa (diluted 1:10 in water, pH 7) for 15-20 minutes. The remaining blood was fixed in absolute ethanol and stored at -20 °C; Whatman FTA Classic cards (GE

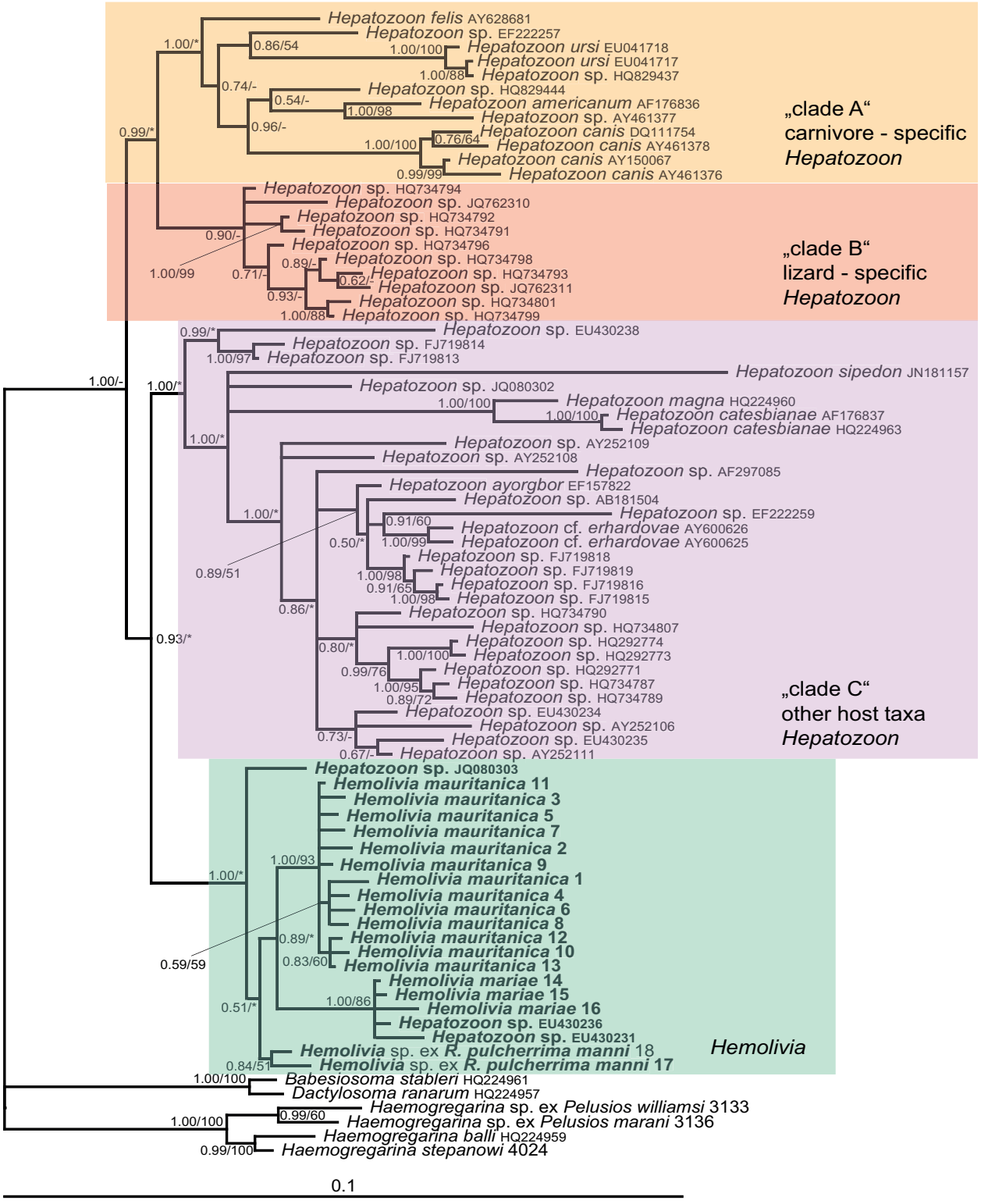

Figure 3. BI and ML tree derived from *Extended matrix*. Numbers at the nodes show posterior probabilities under BI / bootstrap values for ML. Posterior probabilities and bootstrap supports lower than 0.50 or 50 %, respectively, are marked with asterisk (*). BI branches that are not present in ML tree are marked with dash (-).

Table 3. GTR distances obtained for individual clusters.

	Matrix	
	Basic	***Extended***
Hemolivia all	0,020	0,046
Hepatozoon carnivores	0,063	0,058
Hepatozoon Lacertidae	-	0,014
Hepatozoon carnivores + Lacertidae	-	0,058
Hepatozoon others	0,097	0,090
Hepatozoon all	0,103	0,090
Hemolivia and *Hepatozoon* all	0,103	0,200
Babesia	0,144	
Plasmodium	0,162	

Healthcare, Buckinghamshire, UK) were used for the storage of *E. stokesii* blood.

Examination of blood samples, DNA isolation, PCR, sequencing: Stained blood smears were examined by light microscopy using a 100× magnification objective lens equipped with immersion oil. Photomicrographs were taken using an Olympus BX53 microscope equipped with Quick Photo Camera 3.0 PC software. Maximum length and width were recorded for each life-stage. For gamonts and their nuclei, LW (length × width) and L/W (length/width ratio) were also calculated.

Genomic DNA of *Hemolivia* spp. was extracted from the ethanol-preserved blood samples of vertebrate hosts using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturerś instructions. The nuclear 18S rRNA gene was amplified by PCR, using the coccidia-specific primers reported in Kvičerová et al. (2008). All PCR reactions were carried out in 25 μl volume with HotStarTaq DNA Polymerase (Qiagen). The PCR products of expected size (~2000 bp) were enzymatically purified and sequenced, or cloned into the pGEM-T Easy Vector (Promega) and sequenced. Five PCR products or five clones were sequenced for each parasite sample. Sequencing was provided on an automatic 3730XL DNA analyzer in Macrogen, Inc. (Amsterdam, the Netherlands). Obtained sequences were identified by BLAST, edited using the DNASTAR program package (DNASTAR Inc.), and deposited in the NCBI GenBank database under the accession numbers KF992698 - KF992714.

Alignments, phylogenetic analyses: Several different matrices were prepared and analyzed in this study. A *Basic matrix* was constructed to allow for comparison with the preceding study of Barta et al. (2012). This matrix contained all *Hepatozoon* and *Hemolivia* samples from the study of Barta et al. (2012), together with all available *H. mauritanica*, *H. mariae*, and with the sequences of a *Hemolivia* species described above. Only the sequences covering at least 1200 bp long region of 18S rDNA were included in this alignment. For groups of identical sequences, one was arbitrarily chosen as a representative (Table 2, Supplementary Material Table S3). With the addition of a group of lizard-specific samples from the GenBank, we constructed a *Long matrix*. Further, to explore the possible influence of the outgroup composition on the *Hemolivia-Hepatozoon* relationships, we created an *Alternative matrix* extended with phylogenetically distant taxa (i.e. *Adelina*, *Babesia*, *Cryptosporidium*, *Eimeria*, *Klossia* and *Theileria*; see Supplementary Material data Fig. S7). Finally, an *Extended matrix* was constructed by completion of the *Basic matrix* with all the sequences from the publications of Barta et al. (2012) and Maia et al. (2012) regardless of their lengths (see Supplementary Material Table S3).

For all matrices, the sequences were aligned and adjusted manually using BioEdit v. 7.0.5.3. (Hall 1999). To infer phylogenetic relationships, three different approaches were used: Bayesian inference (BI) computed in MrBayes v. 3.2.0. (Huelsenbeck and Ronquist 2001), maximum likelihood (ML) in Phyml v. 2.4.3 (Guindon and Gascuel 2003), and maximum parsimony (MP) in PAUP v. 4.0b10 (Swofford 2001). The best evolutionary models for ML analyses were identified with the jModelTest (Posada 2008, 2009) under the AIC criterion. The trees obtained were visualized and exported using TreeView v. 1.6.6 (Page 1996), and adjusted in Adobe Illustrator CS5 v. 15.0 (Adobe Systems Inc.). See Supplementary data S3 for a list of the sequences used in the phylogenetic analyses.

To estimate the reliability of the phylogenetic arrangement between *Hemolivia* and *Hepatozoon*, we used two additional approaches. The first was based on analyzing a random subset of the *Basic matrix* and comparing the resulting topologies. (The subset yielding different topology in BI and ML is included in the study as a *Monophyletic matrix*). In the second approach, we used the likelihood-ratio-based SH test in PAUP to mutually compare both alternatives. For the *Basic* and *Monophyletic* matrices, we first calculated likelihoods without constraints. The resulting topologies were then used as cross-constraints for the calculation of constrained likelihoods. For each matrix we then evaluated the difference between the likelihoods of unconstrained and constrained topologies using the SH test. To evaluate diversity within *Hemolivia* and the two *Hepatozoon* clades in a broader context, we used PAUP to calculate distances within several clusters under the GTR evolutionary model (see Table 3 for composition of the clusters).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.06.001.

References

Barta JR, Ogedengbe JD, Martin DS, Smith TG (2012) Phylogenetic position of the adeleorinid coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina) inferred using 18S rDNA sequences. J Eukaryot Microbiol **59**: 171–180

Barta JR, Schrenzel MD, Carreno R, Rideout BA (2005) The genus *Atoxoplasma* (Garnham 1950) as a junior objective synonym of the genus *Isospora* (Schneider 1881) species infecting birds and resurrection of *Cystoisospora* (Frenkel 1977) as the correct genus for *Isospora* species infecting mammals. J Parasitol **91**:726–727

Carreno RA, Martin DS, Barta JR (1999) *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol Res **85**:899–904

Desser SS (1993) The Haemogregarinidae and Lankesterellidae. In Kreier JP (ed) Parasitic Protozoa **Vol. 4**, 2nd Edn Academic Press, New York, pp 247–272

Godfrey SS, Bull CM, Murray K, Gardner MG (2006) Transmission mode and distribution of parasite among groups of the social lizard *Egernia stokesii*. Parasitol Res **99**: 223–230

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenesis by maximum likelihood. Syst Biol **52**:696–704

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41**:95–98

Harris DJ, Graciá E, Jorge F, Maia JPMC, Perera A, Carretero MA, Giménez A (2013) Molecular detection of *Hemolivia* (Apicomplexa: Haemogregarinidae) from ticks of North African *Testudo graeca* (Testudines: Testudinidae) and an estimation of their phylogenetic relationships using 18S rRNA sequences. Comp Parasitol **80**:292–296

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**: 754–755

Kvičerová J, Pakandl M, Hypša V (2008) Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. Parasitology **135**: 443–452

Lainson R, De Souza MC, Franco CM (2007) Natural and experimental infection of the lizard *Ameiva ameiva* with *Hemolivia stellata* (Adeleina: Haemogregarinidae) of the toad *Bufo marinus*. Parasite **14**:323–328

Maia JPMC, Perera A, Harris DJ (2012) Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from the western Mediterranean. Folia Parasitol **59**:241–248

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci **12**:357–358

Petit G, Landau I, Baccam D, Lainson R (1990) Description et cycle biologique d'*Hemolivia stellata* n. g., n. sp., hémogrégarine de crapauds Brésiliens. Ann Parasitol Hum Comp **65**:3–15

Pieniazek NJ, Herwaldt BL (1997) Reevaluating the molecular taxonomy: is human-associated *Cyclospora* a mammalian *Eimeria* species? Emerg Infect Dis **3**:381–383

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol **25**:1253–1256

Posada D (2009) Selection of models of DNA evolution with jModelTest. Methods Mol Biol **537**:93–112

Relman DA, Schmidt TM, Gajadhar A, Sogin M, Cross J, Yoder K, Sethabutr O, Echeverria P (1996) Molecular phylogenetic analysis of *Cyclospora*, the human intestinal pathogen, suggests that it is closely related to *Eimeria* species. J Infect Dis **173**:440–445

Schwarz RS, Jenkins MC, Klopp S, Miska KB (2009) Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. J Parasitol **95**:871–880

Sergent Ed, Sergent Et (1904) Sur une hémogrégarine, parasite de *Testudo mauritanica*. C R Soc Biol **56**:130–131

Široký P, Kamler M, Modrý D (2004) Long-term occurrence of *Hemolivia* cf. *mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in captive *Testudo marginata* (Reptilia: Testudinidae): evidence for cyclic merogony? J Parasitol **90**:1391–1393

Široký P, Kamler M, Frye FL, Fictum P, Modrý D (2007) Endogenous development of *Hemolivia mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in the marginated tortoise *Testudo marginata* (Reptilia: Testudinidae): evidence from experimental infection. Folia Parasitol **54**: 13–18

Široký P, Mikulíček P, Jandzík D, Kami H, Mihalca AD, Rouag R, Kamler M, Schneider C, Záruba M, Modrý D

(2009) Co-distribution pattern of a haemogregarine *Hemolivia mauritanica* (Apicomplexa: Haemogregarinidae) and its vector *Hyalomma aegyptium* (Metastigmata: Ixodidae). J Parasitol **95**: 728–733

Smallridge CJ, Bull CM (1999) Transmission of the blood parasite *Hemolivia mariae* between its lizard and tick hosts. Parasitol Res **85**:858–863

Smallridge C, Paperna I (1997) The tick-transmitted haemogregarinid of the Australian sleepy lizard *Tiliqua rugosa* belongs to the genus *Hemolivia*. Parasite **4**: 359–363

Swofford DL (2001) Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA