Multiple host-switching of Haemosporidia parasites in bats
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Abstract

Background: There have been reported cases of host-switching in avian and lizard species of Plasmodium (Apicomplexa, Haemosporidia), as well as in those infecting different primate species. However, no evidence has previously been found for host-swapping between wild birds and mammals.

Methods: This paper presents the results of the sampling of blood parasites of wild-captured bats from Madagascar and Cambodia. The presence of Haemosporidia infection in these animals is confirmed and cytochrome b gene sequences were used to construct a phylogenetic analysis.

Results: Results reveal at least three different and independent Haemosporidia evolutionary histories in three different bat lineages from Madagascar and Cambodia.

Conclusion: Phylogenetic analysis strongly suggests multiple host-switching of Haemosporidia parasites in bats with those from avian and primate hosts.
mals [4]. These parasites are known to be virulent, invasive pathogens in a variety of wild animals and contribute to the parasite burden of natural populations, including several threatened species [5]. Host switching by these parasites could be the trigger for emerging virulent diseases [6-8].

Madagascar and Cambodia are two biodiversity "hot spots" [9]. The faunas of these areas, which are, in evolutionary terms, distant from one another, provide an attractive system for characterizing haemosporidian parasite species [10,11] and for evaluating host-parasite co-evolution and exchange. Madagascar was part to the Gondwana continent and was separated 160 million and 90 million years ago from Africa and from India, respectively; whereas, Cambodia originated from the Laurasia continent.

The paper presents the result of the screening of over 500 bats belonging to seven families from different field sites in Madagascar and Cambodia. Haemosporidia parasites were isolated in the bat families, Hipposideridae, Vespertilionidae and Megadermatidae. Molecular sequences of bat haemosporidian parasites were not previously available. Herein, the phylogenetic analyses with cytochrome b mitochondrial gene sequences illustrate the first documented example of a cross-class host-switching of haemosporidian parasites between birds and mammals.

Methods

Field sites and sample collection

Screened material was obtained in Madagascar and Cambodia. In Madagascar, specimens were obtained at eight different field sites in the provinces of Mahajanga, Toliara and Antananarivo. The fieldwork was conducted between November 2001 and late 2004, in collaboration with the Institut Pasteur de Madagascar and WWF-Madagascar. In Cambodia, two field sites were sampled in the provinces of Kampot and Mondolkiiri between December 2004 and May 2006, in collaboration with the Institut Pasteur du Cambodia and Wildlife Conservation Society. The field research was conducted with local and national permits from the Direction des Eaux et Forêts of Madagascar and the Ministry of Agriculture, Forestry and Fisheries in Cambodia. A total of 530 bats, belonging to seven families (Pteropodidae, Rhinolophidae, Hipposideridae, Megadermatidae, Emballonuridae, Vespertilionidae, and Molossidae) were trapped in the wild. A small sample of blood was obtained and the host was released without injury or in some cases retained as a voucher specimen.

Microscopic examination

Thin blood smears were made for each animal. They were fixed in methanol and stained by incubation with 10% Giemsa for 10 minutes. The smears were examined by microscopy for haemosporidian parasites. Parasites isolated from bats were referred to as Haemosporidia sp. Positive blood smears were catalogued in the Département 'Régulations, Développement et Diversité Moléculaire', Muséum National d'Histoire Naturelle, Paris, France.

DNA extraction and amplification

DNA was extracted from blood samples using the phenol/chloroform technique [12]. The 709 bp cytochrome b fragments were amplified using PCR and nested-PCR. The PCR reaction was carried out in a total volume of 25 µl under the following condition: 1 µl of each of the primers: PLAS 1 (5'-GAGAATTATGGGATGATGGTG-3') and PLAS 2 (5'-GTGGTAATTGACATCCWATCC-3'), 1 mM of each dNTP, 1 U of Taq polymerase (Solis), 3 mM MgCl₂. The PCR conditions were: 5 min at 94°C, 30 sec at 94°C, 30 sec at 55°C and 1 min 30 sec at 72°C for 40 cycles and a final 10 min extension at 72°C. The nested-PCR was carried out using 1 µl of the PCR products and performed with the following primers: PLAS 3 (5'-GGTGTITYIAGATAYATGCAYGC-3') and PLAS 4 (5'-CATCWWATCCTAR-TAWAGCATAG-3'). The conditions were: 5 min at 94°C, 30 sec at 94°C, 30 sec at 55°C, 1 min 30 sec at 72°C for 40 cycles and a final 10 min extension at 72°C. The PCR products were sequenced using PLAS3 and PLAS4 primers by Macrogen (Korea). All of these primers were designed by the research group. They are specific to haemosporidian parasites and do not amplify others Apicomplexa parasites or host DNA. Relevant sequences were selected for the phylogenetic analysis. There are numerous avian parasite sequences available and some representative sequences were chosen based on geographical localizations.

Phylogenetic analyses

The nucleotide sequences (709 bp) were translated into amino acid sequences to minimize homoplasly due to saturation of synonymous mutations since some taxa have diverged over hundreds of millions years. The sequences were aligned using CLUSTAL W [13]. Reference sequences of at least 219 amino acids without ambiguous positions were retrieved from GenBank. In the case of identical sequences in amino acids, only one sequence for the analysis was kept. Maximum Likelihood (ML) was performed using Phylip [14] and Parsimony analysis (P) was performed using Phylip package (version 3.62) [15]. Nodal robustness was evaluated by non-parametric bootstrap (100 replicates). Bayesian analyses were conducted with MrBayes V3.1 software [16] using gamma distribution, 1 000 000 generations (average standard deviation of split sequences is below 0.01), with tree sampling every 100 generations and a burn-in of 2500 trees. The aim of the study was to analyse Haemosporidia phylogeny, so Thileeria annulata, Babesia gibsoni and Toxoplasma gondii which belong to the phylum Apicomplexa but are non-haemos-
poridian parasites were choose as out-groups. The phylo-
genetic study was run using cytochrome $b$ sequences. This
is the most abundant marker in GenBank for a variety of
haemosporidian parasites and widely used in phyloge-
netic studies because reference sequences from other
genes are lacking.

**Results**

530 blood samples were collected in Madagascar and in
Cambodia belonging to seven families of bats. Haemos-
poridian infections were identified in three families: Hip-
posideridae, Vespertilionidae and Megadermatidae (Table
1 and Table 2). All the cytochrome $b$ sequences that were
isolated from these bats were previously unpublished and
the parasite taxa, the host names, the collection localities
and the GenBank accession numbers are given in Table 3.
The three phylogenetic methods used, Parsimony (P),
Maximum Likelihood (ML) and Bayesian analyses pro-
duced the same tree topology. The phylogeny is presented
in Figure 1.

### Table 1: Prevalence of Haemosporidia infection in bats from Madagascar

| Species                  | Family           | n sampled | n infected (%) |
|--------------------------|------------------|-----------|----------------|
| Eidolon dupreanum*       | Pteropodidae     | 3         | 0              |
| Rousettus madagascariensis* | Pteropodidae   | 33        | 0              |
| Hipposideros commersoni  | Hipposideridae   | 23        | 0              |
| Triaenops furculis*      | Hipposideridae   | 28        | 1 (4)          |
| Emballonura tiavata*     | Emballonuridae   | 41        | 0              |
| Miniopterus gleni        | Vespertilionidae | 2         | 0              |
| Myotis goudoti           | Vespertilionidae | 13        | 3 (23)         |
| Miniopterus manavi*      | Vespertilionidae | 68        | 16 (24)        |
| Chaerophon sp.*          | Molossidae       | 129       | 49 (38)        |
| Mormoproteus jugularis*  | Molossidae       | 1         | 0              |
| Otomops madagascariensis*| Molossidae       | 25        | 0              |
| Chaerophon leucogaster*  | Molossidae       | 23        | 0              |
| Mops leucostigma         | Molossidae       | 9         | 0              |

* endemic species

### Table 2: Prevalence of Haemosporidia infection in bats from Cambodia

| Species                  | Family           | n sampled | n infected (%) |
|--------------------------|------------------|-----------|----------------|
| Rhinolophus acuminatus   | Rhinolophidae    | 3         | 0              |
| Rhinolophus borneensis   | Rhinolophidae    | 3         | 0              |
| Rhinolophus malayanus    | Rhinolophidae    | 1         | 0              |
| Rhinolophus shameli      | Rhinolophidae    | 2         | 0              |
| Rhinolophus sp.          | Rhinolophidae    | 1         | 0              |
| Hipposideros amniger     | Hipposideridae   | 2         | 0              |
| Hipposideros larvatus    | Hipposideridae   | 13        | 1 (8)          |
| Hipposideros pomona      | Hipposideridae   | 1         | 0              |
| Megaderma spasma         | Megadermatidae   | 5         | 4 (80)         |
| Taphozous melanopogon    | Emballonuridae   | 19        | 0              |
| Glischropus sp.          | Vespertilionidae | 3         | 0              |
| Murina cyclotis          | Vespertilionidae | 4         | 0              |
| Murina tubinaris         | Vespertilionidae | 6         | 0              |
| Myotis microspheorus    | Vespertilionidae | 1         | 0              |
| Kerivoula hardwickii    | Vespertilionidae | 10        | 2 (20)         |
| Kerivoula papillosa      | Vespertilionidae | 4         | 0              |
| Tylonycteris robustula   | Vespertilionidae | 7         | 0              |
| Chaerophon plicatus      | Molossidae       | 5         | 0              |

Total 90 7
The results show the existence of two clades within Haemosporidia, separating mammal and sauropsid hosts (birds and lizards) (1.00 Bayesian posterior probabilities, 99 and 100 for ML and P respectively bootstrap support). In the first clade, the four malaria parasites afflicting humans, Plasmodium malariae, Plasmodium ovale Plasmodium vivax and P. falciparum form a polyphyletic group [17]. Rodent Plasmodium are the sister group and P. falciparum still exhibits a deep branch. Interestingly, parasite isolated from the bat Hipposideros larvatus (Family Hipposideridae) (Figures 2 and 3) clusters with a Hepatocystis parasite obtained from a baboon (Papio sp.) and falls within the Plasmodium primate group (Bayesian posterior probabilities of 1.00 and bootstrap support of 99 and 96 for ML and P, respectively).

In the second clade, between two clades of *Plasmodium*, are *Leucocytozoon* and *Haemoproteus*, two genera infecting only sauropsid hosts, and are close sister taxa of bird and lizard *Plasmodium* [18]. This renders the genus *Plasmodium*

### Table 3: Parasite taxa used in this study with host name, geographic location and GenBank accession number of the sequences used for the phylogenetic analysis

| Parasites          | Host                        | Geographic locality         | GenBank accession number |
|--------------------|-----------------------------|-----------------------------|--------------------------|
| Plasmodium falciparum | Homo sapiens                | Tropical regions            | AY069605                 |
| Plasmodium gonderi  | Old world monkeys           | Central Africa              | AF069622                 |
| Plasmodium inui     | Old world monkeys           | India and southeast Asia    | AF069617                 |
| Plasmodium knowlesi | Old world monkeys           | Malaysia                    | AF069621                 |
| Plasmodium malariae | Homo sapiens                | Tropical and subtropical regions | AF069624          |
| Plasmodium ovale    | Homo sapiens                | Tropics of Africa and Asia  | AF069625                 |
| Plasmodium vivax    | Homo sapiens                | Tropical and subtropical regions | AF069619          |
| Hepatocystis sp.    | Papio sp.                   | Ethiopia                    | AF069626                 |
| Plasmodium sp.      | Mandrillus leucophaeus      | Gabon                       | AF069623                 |
| Plasmodium athereri | Atherurus africanus         | Congo and Cameroon          | AY099054                 |
| Plasmodium berghii  | Grammomys surdaster        | Central Africa              | AF099049                 |
| Plasmodium chabaudi | Thamnomys rutulans         | Central Africa              | AF099050                 |
| Plasmodium vinckei  | Grammomys surdaster        | Congo                       | AF099052                 |
| Plasmodium elongatum| Passer domesticus           | North America               | AY069611                 |
| Plasmodium gallinaceum | Gallus gallus             | Vietnam                     | DQ212189                 |
| Plasmodium relictum | Zeneida macroura           | North America               | AY099032                 |
| Plasmodium sp. 2    | Ninax scutulata             | Singapore                   | AY099035                 |
| Plasmodium sp. 1    | Acrocephalus arundinaceus   | Japan                       | AY099044                 |
| Plasmodium juxtanucleare | Gallus gallus            | Asia                        | AB250415                 |
| Plasmodium catheremeri | Wide range Bird         | Wallacean zones             | AY377128                 |
| Plasmodium sp. 47   | Agelaus phoeniceus         | North America               | AF465547                 |
| Plasmodium sp. 49   | Zonotrichia leucophrys     | North America               | AF465549                 |
| Plasmodium sp. 50   | Andropadus latirostris     | Cameroon                    | AF465550                 |
| Plasmodium floridense | Anolis acutus             | Dominica                    | AY099059                 |
| Plasmodium mexicanum | Sceloporus occidentalis   | California                  | AY099060                 |
| Plasmodium chironius | Sceloporus jarrovi         | Arizona                     | AY099061                 |
| Haemoprotea (FMNH 172853) | Miniopterus manavi    | Madagascar                  | AY762070*                |
| Haemoprotea (FMNH 172862) | Miniopterus manavi    | Madagascar                  | AY762071*                |
| Haemoprotea (FMNH 172918) | Miniopterus manavi    | Madagascar                  | AY762074*                |
| Haemoprotea (FMNH 175810) | Myotis goudoti         | Madagascar                  | AY762075*                |
| Haemoprotea (C285)  | Kerivoula hardwickii      | Cambodia                    | EF179354*                |
| Haemoprotea (C289)  | Megadema spasma           | Cambodia                    | EF179355*                |
| Haemoprotea (C272)  | Hipposideros larvatus     | Cambodia                    | EF179356*                |
| Haemoproteus majoris | Parus caeruleus           | Sweden                      | AY099045                 |
| Haemoproteus sylvae | Acrocephalus arundinaceus | Sweden                      | AY099040                 |
| Haemoproteus sp. 1  | Phylloscopus occipitalis   | India                       | AY099043                 |
| Haemoproteus sp. 2  | Phylloscopus occipitalis   | India                       | AY099043                 |
| Haemoproteus sp. 3  | Acrocephalus scirpaceus   | Spain                       | AY099046                 |
| Leucocytozoon toddi | Accipiter francesii       | Madagascar                  | AY684973                 |
| Toxoplasma gondii   |                            |                            | AY023246                 |
| Theileria annulata  |                            |                            | M63015                   |
| Babesia gibsoni     |                            |                            | AB215096                 |

FMNH: Field Museum of Natural History
C: Cambodia
* Previously unpublished sequences
polyphyletic. Unexpectedly, the haemosporidian parasite isolated from the bat Megaderma spasma (Family Megadermatidae) (Figure 4) is included within the sauropsid Plasmodium clade. However, the bootstrap value is low (Bayesian posterior probabilities of 0.62). Remarkably, haemosporidian parasites isolated from the Malagasy endemic bats Myotis goudoti and Miniopterus manavi and the parasite isolated from the widespread Asiatic bat Kerivoula hardwickii (Figure 5) form a monophyletic cluster and fall within the sauropsid Plasmodium clade. All these bats are classically placed in the Family Vespertilionidae. This result is well support with 0.75 Bayesian posterior probabilities and 75 ML bootstrap.

Discussion
The mitochondrial genome is more conserved in apicomplexan parasites [19] than in others metazoan eukaryotes. Cytochrome b gene is, therefore, a good marker to establish phylogenetic relationships between parasites that diverged several millions years ago [18].

The presence of a major division within haemosporidian parasites separating mammal and sauropsid hosts, suggests that parasites of these two vertebrate groups evolved separately. In the mammalian clade of parasites, except for P. falciparum, all primate Plasmodium species share a common ancestor, although host shifts occurred during the course of primate speciation. Indeed, wild primate populations are potential reservoirs for human malaria parasites [8] and host shifts have occurred in Southeast Asia with, for example, Plasmodium knowlesi, which usually infect macaques, afflicting humans [20]. Recently evidence on the origin of P. vivax as a macaque monkey malaria parasite in Southeast Asia has been proposed

Figure 1
Phylogeny of Haemosporidia inferred from cytochrome b amino acid sequences. Value above branches are Bayesian posterior probabilities [16] (value less then 0.5 not shown), below are bootstrap percentage obtained by maximum likelihood [14] (left of the slash, values under 50% not shown). In red are the previously unpublished bat sequences. See Tables 1 and 2 for sampling details. H. = Haemoproteus, L. = Leucocytozoon and P. = Plasmodium.
In addition, *Plasmodium simium* and *Plasmodium brasilianum*, two species infecting South American platyrhini primates, are genetically indistinguishable from *P. vivax* and *P. malariae*, respectively, and may have been associated with a human-platyrrhini host-switch [8,21]. Further, haemosporidian parasite isolated from *Hipposideros larvatus* clusters with a baboon *Hepatocystis*. This association between bat and primate parasites has been previously proposed based on mitochondrial data, where a *Hepatocystis* species isolated from a bat (*Cynopterus brachyotis*, Family Pteropodidae) clusters with this baboon *Hepatocystis* [4]. Needless to say, this result is not congruent with mammal phylogeny [22] and suggests a host switch from primates to bats.

In the sauropsid clade, the evolutionary history of *Plasmodium* that infects birds and lizards is not resolved. The parasite phylogeny clearly does not fit with the host phylogeny. *Plasmodium* parasites from birds and lizards are known to show little host specificity [23]. Previous conclusions support that infrequent and unpredictable host shifts have occurred in the parasite-host sauropsid system [24]. Surprisingly, the Haemosporidia isolated from *Megaderma spasma* in Cambodia falls within the sau-
rhopid Plasmodium clade. However, phylogenetic relationships between the parasites of Megaderma and sauropsids are not completely resolved.

Furthermore, closely related haemosporidian parasites isolated from Myotis goudoti and Miniopterus manavi, two endemic Malagasy bat species and the haemosporidian parasite from the Cambodian Kerivoula hardwickii, all of which are placed in the family Vespertilionidae, fall within the sauropsid Plasmodium clade. This result clearly does not fit with vertebrate phylogeny and supports host switching from birds to bats. The haemosporidian vespertilionid parasites from Madagascar and Cambodia are monophyletic, which suggest that the host switching took place in the early evolutionary history of these bats and was followed by subsequent radiation and co-speciation. This is the first report showing host switching in haemosporidian parasites between birds and mammals (bats).

After rodents, bats are the largest order of mammals (at least 1,100 species, more than 20% of extant mammal species). The Chiroptera are very diverse and they are distributed almost worldwide and have extremely diverse life history traits and morphology. Based on recent molecular work, they are classified into four super-families that apparently diversified in different areas during the early Eocene as a "Big Bang" radiation [25] coincident with the peak of Tertiary insect diversity [26]. In developing echolocation and different flight strategies, the ancestors of modern bats colonized various ecological niches [27], where birds and their associated blood parasites are thought to have been present, thus favoring host switching from birds to bats. Furthermore, Myotis goudoti and Miniopterus manavi often share common day roost sites in tree hollows, caves and rock shelters [28], which expose considerable numbers of densely packed individuals to the same potential blood parasite vectors.

Conclusion

The introduction of the 7 new genetic sequences from chiropteran hemoparasites does not alter the deep branching of P. falciparum within the mammalian clade [4,17]. This result does not support a recent parasite transfer from bats to birds. Furthermore, closely related haemosporidian parasites isolated from Myotis goudoti and Miniopterus manavi, two endemic Malagasy bat species and the haemosporidian parasite from the Cambodian Kerivoula hardwickii, all of which are placed in the family Vespertilionidae, fall within the sauropsid Plasmodium clade. This result clearly does not fit with vertebrate phylogeny and supports host switching from birds to bats. Furthermore, closely related haemosporidian parasites isolated from Myotis goudoti and Miniopterus manavi often share common day roost sites in tree hollows, caves and rock shelters [28], which expose considerable numbers of densely packed individuals to the same potential blood parasite vectors.

Evidence presented herein of host switching between birds and bats, it is difficult to reject the hypothesis that P. falciparum has a non-primate origin. It may have emerged in humans associated with an ancient host switching and, as such, it could be one of the oldest "emerging" diseases in humans.

Authors' contributions

LD, VR, and FA designed the study. LD, VR, GC, MR, JW, TN, SMG and FA do the sampling. LD and TN process samples and LD, HA, VR, SMG and FA analysed the data. LD wrote the first draft of the manuscript then VR, SMG and FA critically reviewed the manuscript. All authors read and approved the final manuscript.

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