



# First evidence for development of *Plasmodium relictum* (Grassi and Feletti, 1891) sporozoites in the salivary glands of *Culex modestus* Ficalbi, 1889

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Received: 21 November 2022 / Accepted: 18 April 2023 / Published online: 26 April 2023  
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## Abstract

The competence of insect vectors to transmit diseases plays a key role in host-parasite interactions and in the dynamics of avian malaria and other haemosporidian infections (Apicomplexa, Haemosporida). However, the presence of parasite DNA in the body of blood-sucking insects does not always constitute evidence for their competence as vectors. In this study, we investigate the susceptibility of wild-caught mosquitoes (*Culex* spp.) to complete sporogony of *Plasmodium relictum* (*cyt b* lineage SGS1) isolated from great tits (*Parus major* L., 1758). Adult female mosquitoes were collected with a CO<sub>2</sub> bait trap overnight. A set of 50 mosquitoes was allowed to feed for 3 h at night on a single great tit infected with *P. relictum*. This trial was repeated on 6 different birds. The bloodfed mosquitoes that survived ( $n = 68$ ) were dissected within 1–2 days (for ookinetes,  $n = 10$ ) and 10–33 days post infection (for oocysts and sporozoites,  $n = 58$ ) in order to confirm the respective parasite stages in their organs. The experiment confirmed the successful development of *P. relictum* (*cyt b* lineage SGS1) to the stage of sporozoites in *Culex pipiens* L., 1758 ( $n = 27$ ) and in *Culex modestus* ( $n = 2$ ). Our study provides the first evidence that *C. modestus* is a competent vector of *P. relictum* isolated from great tits, suggesting that this mosquito species could also play a role in the natural transmission of avian malaria.

**Keywords** Avian malaria · Blood parasites · Competent vector · *Parus major* · SGS1 · Transmission

## Introduction

Vector-transmitted diseases such as avian malaria (*Plasmodium* spp.) might play an important role in adaptation and natural selection in bird populations (Atkinson et al. 2013). These parasites are widespread and highly prevalent among birds and can have a significant influence on host wellness and survival (Valkiūnas 2005). Although insect vectors play a key role in avian malaria transmission and dynamics, they are often neglected in ecological studies and in analyses of host-parasite interactions (Martínez-de la Puente et al. 2018, 2020).

The species of the genus *Plasmodium* are transmitted by vectors of the family Culicidae (Diptera), in which sexual process and sporogony take place

(Santiago-Alarcon et al. 2012). Successful development of sporozoites in the blood-feeding insects is an obligatory criterion to recognize a certain insect species as a competent vector of a certain parasite species (Valkiūnas et al. 2013). Recently, many surveys are based mostly on molecular detection of parasite DNA in mosquitoes' whole body, abdomen (filled/not filled with blood) and head and thorax (Zélé et al. 2014; Martínez-de la Puente et al. 2015; Schoener et al. 2017; Guimarães et al. 2021). Though these studies involve molecular identification of the blood source and comparison of the parasite lineages found in particular host species, they mostly draw conclusions about “potential vectors” (Schoener et al. 2017; Guimarães et al. 2021). The currently used PCR-based methods cannot distinguish between different stages of parasite development in mosquitoes and would score as positive both presence of gametocytes, (or gametes, ookinetes, and oocysts) and presence of sporozoites. A combination of microscopic examination of salivary glands for sporozoites and PCR approach for identification of parasite lineages is essential in order to

Section Editor: Helge Kampen

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reveal competent vectors (Kim and Tsuda 2015). There are few experimental studies documenting the development of parasite and proving complete sporogony in a vector species (Kazlauskienė et al. 2013; Valkiūnas et al. 2015; Gutiérrez-López et al. 2016).

In the present study, we investigated the ability of wild mosquitoes (*Culex* spp.) to support complete sporogonic development of the widespread malaria parasite, *Plasmodium relictum* mitochondrial cytochrome b (*cyt b*) lineage SGS1, isolated from naturally infected great tits (*Parus major*).

## Materials and methods

### Insect collecting

Female mosquitoes were collected overnight in August 2017 at Kalimok Biological Station, northeast Bulgaria (44.01142° N, 26.43965° E) in two types of habitats: planted deciduous forest and reedbeds. We used All Weather EVS-CO<sub>2</sub> Mosquito Trap (BioQuip Products, Inc.) with compressed bottle CO<sub>2</sub>, adjusting the CO<sub>2</sub> volume with CO<sub>2</sub> upgrade set (Biogents AG) to 0.5 kg per day. In the morning, the collected mosquitoes were moved to an insectarium and kept there for 2–3 days until the start of the experiments ( $t = 23\text{--}25$  °C, humidity over 60%, 12-h light-and-dark cycle).

### Experimental setup and insect dissection

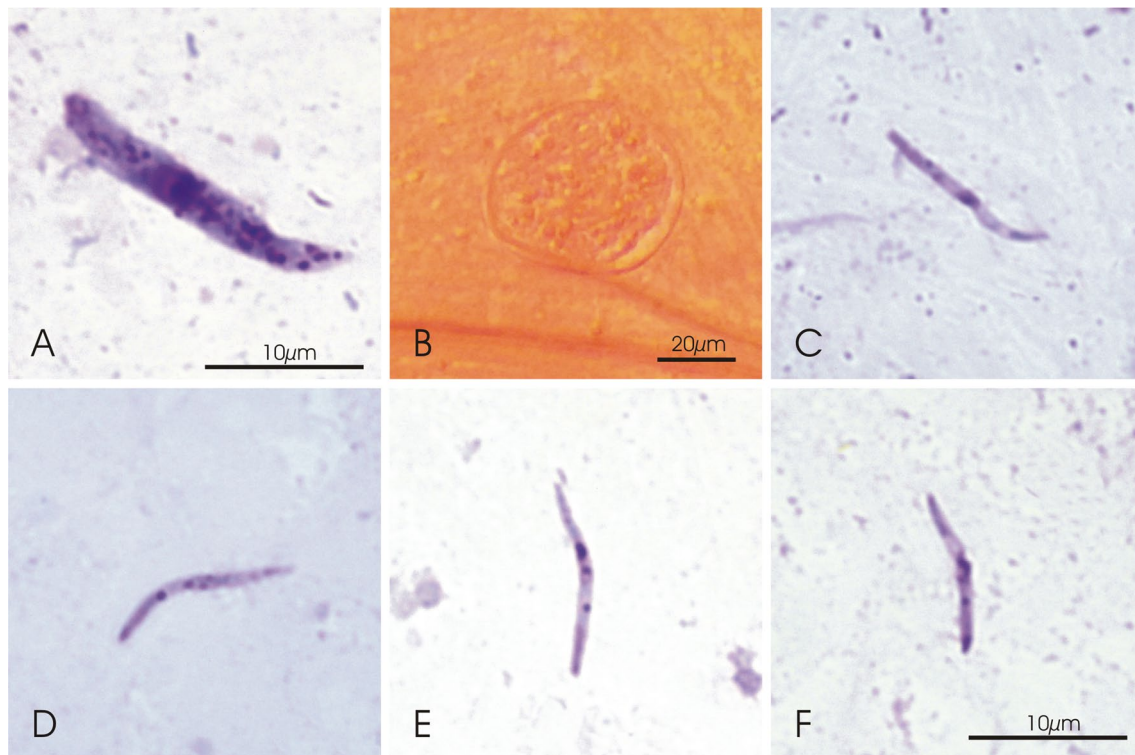
A set of 50 female mosquitoes was allowed to feed for 3 h at night on a single great tit infected with *P. relictum*, *cyt b* lineage SGS1. This trial was repeated on a total of 6 birds infected with *P. relictum*. Two blood smears and one blood sample in SET buffer (0.05 M tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) were collected after the puncture of the brachial vein of the birds. Parasitaemia and gametocytaemia of the infected birds were estimated as percent of the infected erythrocytes (actual count of 10,000 erythrocytes when parasitemia is < 0.1% and 1000 erythrocytes when parasitemia is > 0.1%) on Giemsa-stained thin blood films.

Birds were placed in individual cages (size 60 cm × 60 cm × 80 cm) in complete darkness and mosquitoes were released with a remote pneumatic system into the cage after the birds had calmed down and had become still on the perches. Three hours after the onset of the trial, the great tits were caught and removed from the cages, still in the dark. The survived mosquitoes were

collected the next morning with an insect aspirator to examine the development of the parasitic stages in their organs. The bloodfed mosquitoes ( $n = 68$ ) were dissected either within 1–2 days post-infection (dpi) for examination of development of ookinets ( $n = 10$ ) or within 10–33 dpi for detection of oocysts and sporozoites ( $n = 58$ ). Prior to dissection, the mosquitoes were anesthetized with a cotton pad soaked with 95% ethanol. Afterwards, each mosquito was placed on a clean glass slide under a stereo microscope (Ceti Steddy-BR). Dissection started by removing the legs and wings of the mosquito and storing them in a tube with 99% ethanol. A sterile syringe needle, pincers, and an entomological needle were used to extract the midgut and salivary glands in a drop of saline. The preparations for ookinete and sporozoite examination were made in a drop of saline on a glass slide, air-dried, fixed in absolute methanol, and stained in 10% Giemsa solution for 1 h. Temporary preparations for oocysts were prepared in a drop of saline, covered with coverslip, and stained with 2% mercurochrome solution. All the mosquito remains were placed in a tube with 99% ethanol for molecular identification of parasite lineages and insect species. All preparations were examined under light microscopes (Ceti Magnum Binocular Compound and Zeiss Axio Imager M2) with 400 × and 1000 × magnification.

### Molecular identification of mosquito species

Mosquitoes that had sporozoites in the salivary glands were identified molecularly. Total DNA was extracted from the mosquito remains via PureLink™ Genomic DNA Mini Kit (Invitrogen™), following the manufacturer's instructions. Universal primer pair LCO1490 and HCO 2198 (Folmer et al. 1994) was used to amplify a 658 bp fragment of insect mitochondrial COI gene in the following temperature regime: 1 min at 94 °C; five cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1 min at 72 °C and a final cycle of 5 min at 72 °C (Hebert et al. 2003). PCR reaction was performed by using Thermo Scientific DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc). All amplified fragments were sequenced on both strands at MacroGen Ltd. (Amsterdam, the Netherlands). Obtained sequences were edited, assembled, and aligned using CodonCode Aligner version 8.0.2 (CodonCode, Dedham, MA, USA) and compared to the available sequences in the GenBank DNA database.



**Fig. 1** Ookinete (A), oocyst (B), and sporozoites (C–F) of *Plasmodium relictum*, *cyt b* lineage SGS1 developed in *Culex pipiens* (A–D) and *Culex modestus* (E, F)

### Molecular identification of *Plasmodium* spp.

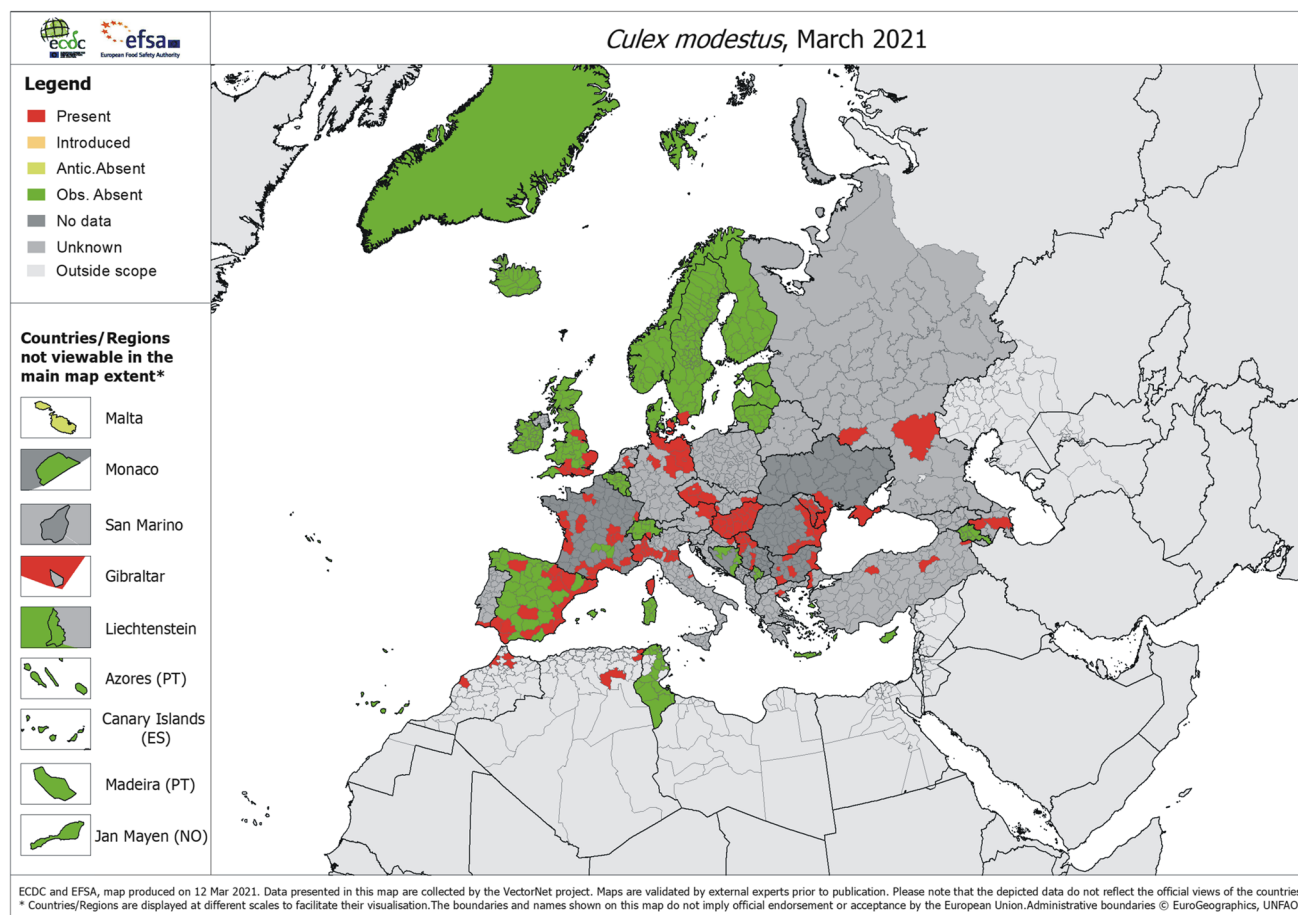
Genomic DNA was used for the amplification of 479 bp fragment of mitochondrial *cyt b* gene of the haemsporidian parasites as described by Hellgren et al. (2004). The reaction was carried out using Thermo Scientific DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc). Presence of amplicons was visualized on 2% agarose gel stained with GelRed (Biotium Inc.). All samples positive for parasites were sequenced at MacroGen Ltd. (Amsterdam, the Netherlands) from the 5' end with the primer HaemF (ATGGTGCTTTCGATATATGCATG). Obtained sequences were edited, assembled, and aligned using CodonCode Aligner version 8.0.2 (CodonCode, Dedham, MA, USA) and compared to the available sequences in the MalAvi database (Bensch et al. 2009).

### Results and discussion

Out of the 68 dissected mosquitoes, 29 were confirmed to have a complete sporogony of *P. relictum* (*cyt b* lineage SGS1) by detecting sporozoites in the salivary glands (Fig. 1C–F) and by PCR amplification and sequencing parasite DNA. Of the 29 mosquitoes,

27 were molecularly identified as *Culex pipiens* and 2 as *Culex modestus*. The morphological features and size of the sporozoites were similar in *C. pipiens* (Fig. 1C, D) and in *C. modestus* (Fig. 1E, F). On the day of exposure, parasitemia of the donors (great tits) varied between 0.01 and 22.00%. The range of gametocytaemia was between 0.01 and 0.90%. Ookinetes were observed in 24 hrs post infection in two out of ten dissected mosquitoes (Fig. 1A). Oocysts were observed within 11–33 days post infection (dpi) with mean abundance 5.5 (0–46,  $n = 37$ ) (Fig. 1B). A positive correlation between parasitaemia and the number of oocysts was found (Spearman rank order correlation  $r_s = 0.6$ ,  $p = 0.00007$ ,  $n = 37$ ). Accordingly, parasitemia have been considered as good predictor for oocyst burden in *C. pipiens* (Pigeault et al. 2015).

*Culex pipiens* is the most abundant ornithophilic mosquito at the study site and is likely to play a significant role in spreading avian malaria among birds in the area (Bobeve et al. unpubl.). The less common *C. modestus* was found for the first time to be a competent vector of *P. relictum*, which was able to complete sporogony and to develop sporozoites in this mosquito species. A survey of avian malaria in *Culex* mosquitoes in south Spain showed the presence of *P. relictum* lineage SGS1 in *C. modestus* head and thorax



**Fig. 2** Distribution of *Culex modestus* in Europe and North Africa. European Centre for Disease Prevention and Control and European Food Safety Authority. Mosquito maps [internet]. Stockholm: ECDC;

2021. Available from: <https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps>

(Ferraguti et al. 2013) but did not confirm sporozoite development.

*Culex modestus* has a patchy distribution and is mostly found in central and southern Europe (Fig. 2). It seems that *C. modestus* prefers less urbanized wetland areas (Ferraguti et al. 2016). Similarly, this species was mostly caught in wetland reedbeds at Kalimok Station. *Culex modestus* has been studied as main competent vector for West Nile virus in France (Balenghien et al. 2007). From the present results, this mosquito might as well participate in spreading avian malaria, more specifically *P. relictum* SGS1 lineage, among birds inhabiting mesic habitats. Its significance as a vector of avian malaria remains to be assessed by collecting further data of its seasonal abundance as well as host and habitat preference.

Our study also confirms the complete sporogony of *P. relictum* (*cyt b* lineage SGS1) in *C. pipiens*, as revealed in previous studies (Kazlauskienė et al. 2013). *Plasmodium relictum* (lineage SGS1) is a globally spread host-generalist

parasite found in 139 bird species of 11 orders inhabiting Eurasia, Africa, North and South America, and Oceania (Bensch et al. 2009). Our findings bring new knowledge about the vector species responsible for the transmission of this most prevalent and widespread avian malaria parasite.

**Acknowledgments** We are grateful to Dr. Chiara Marchetti and Prof. Boyko Georgiev for their valuable comments on the English language and to both anonymous reviewers and the Section Editor for their constructive comments and suggestions on the manuscript. We thank Zuberia Ismail and Ljatif Ismail for their help with the fieldwork.

**Data availability** All permanent preparations and raw data can be provided upon request.

**Author contributions** Dimitar Dimitrov, Martin Petrov Marinov, and Pavel Zehindjiev designed the study; Dimitar Dimitrov and Martin Petrov Marinov performed the fieldwork and the experiments; Dimitar Dimitrov did dissections of the insects and microscopic examination; Aneliya Bobeva organized and performed the molecular lab work. Mihaela Ilieva and Dimitar Dimitrov drafted the manuscript. All authors contributed with final review of the manuscript.

**Funding** The study was funded by the Bulgarian National Science Fund (DN 01/6).

## Declarations

**Ethics approval** The capture and maintaining of the animals correspond to the current legislation in Bulgaria under permission no. 672/17.03.2016, issued by the Ministry of Environment and Water.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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