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FOUR NEW SPECIES OF *PLASMODIUM* FROM NEW GUINEA LIZARDS: INTEGRATING MORPHOLOGY AND MOLECULES

Susan L. Perkins and Christopher C. Austin*

Sackler Institute for Comparative Genomics & Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th St., New York, New York 10024. *e-mail: perkins@amnh.org*

ABSTRACT: New Guinea is one of the most biodiverse regions of the world, particularly in terms of the herpetofauna present, yet surprisingly little is known about the parasites that infect these organisms. A survey of diverse scinid and agamid lizard hosts from this country showed a diversity of malaria parasites infecting these hosts. We combined morphological and morphometric observations of the parasites (primarily gametocytes) along with DNA sequence data from the mitochondrial cytochrome *b* and cytochrome oxidase I genes and here describe 4 new species of *Plasmodium*, i.e. *Plasmodium minuoviride* n. sp., *Plasmodium koreafense* n. sp., *Plasmodium megalotrypa* n. sp., and *Plasmodium gemini* n. sp. A fifth species, *Plasmodium lacertiliae* Thompson and Hart 1946, is redescribed based on new observations of hosts and localities and additional molecular data. This combined morphological and molecular approach is advised for all future descriptions of new malaria parasite species, particularly in light of situations where every life-history stage is not available.

Approximately 200 species of *Plasmodium* have been described from mammal, avian, and squamate reptile intermediate hosts. These species descriptions have relied on classical methods such as morphological characters, life-history traits, and host taxon, all of which are useful, but all of which are also subject to serious shortcomings. For example, appearance of the parasites on thin blood films can be altered by the rate of drying of the original material and staining protocol. Also, characteristics long held as definitive for the classification of parasites into genera and subgenera, including the production of pigment and the presence of erythrocytic schizonts, are now thought to be considerably more plastic. Examples of this include the monophyly of parasites such as *Plasmodium azurophilum* Telford, 1975, a lizard parasite that does not produce pigment, and *Hepatocystis* species, mammalian parasites that do not have erythrocytic schizonts, with other species of *Plasmodium*, which is defined with the characters of pigment production and erythrocytic schizogony (Perkins and Schall, 2002; Martinsen et al., 2006, 2008). Ideally, to describe a species of *Plasmodium*, data provided would include morphology of all stages, in both the vertebrate and insect hosts, from multiple infections, as well as information on the complete life cycle (timing of all developmental stages), and the full range of host and vector species. Very few *Plasmodium* species have all of this kind of information available, and those that do are primarily those species that infect humans or the mammalian parasites that are model organisms. In many cases, only a few—and sometimes just a single—blood smears have been available when defining new species; this is clearly far from the ideal method.

For various reasons, sometimes the acquisition and study of all parasite life stages, or even all of those typically used for species descriptions of *Plasmodium* species, are not possible. Many malaria parasites, from those infecting humans (e.g., Hamad et al., 2002; Drakeley et al., 2006) to those infecting birds (Cosgrove et al., 2008) and lizards (Goodwin and Stapleton, 1952; Jordan and Friend, 1971; Bromwich and Schall, 1986), show marked seasonality, where there is an almost complete absence of various life-history stages at certain points of the year. Thus, blood samples of vertebrates during typical field collections may reveal the presence of parasites, but often not in all life stages. Still, the taxonomic description of these parasites as species is valuable as a means of quantifying biodiversity and providing a framework within which additional study, including more detailed studies of the life cycle, can be performed. Until recently, however, a means with which to link samples from multiple hosts taken at different times has not been possible.

The use of DNA sequences has recently started to serve as a powerful bridge to allow for the diagnosis of species when they are present in different life-history stages, i.e., the eggs of fish (Desalle and Birstein, 1996), larval and adult arthropods (Barber and Boyce, 2006; Ahrens et al., 2007; Zhou et al., 2007), and efficient diagnosis and study of parasites with complex life cycles (Brant et al., 2006). The iterative process of sequencing a gene or genes from parasites, the phylogenetic study of those sequences in the context of other detected infections and samples of previously described species, and then the deposition of these sequences into databases can provide an efficient and cohesive process for the taxonomic study of parasites. DNA sequences should always be coupled with morphological observations of at least some of the life-history stages so that independent lines of evidence are available for species identity and description (DeSalle et al., 2005).

In this study we conducted a large survey of the lizards, primarily skinks, from several sites in one of the most biologically diverse regions of the world, Papua New Guinea, taking blood smears and tissue collections from each. New Guinea has been recognized as one of the world's 5 'High Biodiversity Wilderness Areas,' and, as the world's largest and tallest tropical island, it represents the third largest tract of intact rainforest after the Amazon and Congo basins (Austin, 1995; Mittermeier et al., 2003). The island occupies less than 0.6% of global land area, but it is estimated to harbor 5–7% of the world's biodiversity (Beehler, 1993; Dinerstein and Wikramanayake, 1993; Mack, 1998; Myers et al., 2000). Much of this estimated diversity is not known to science. In particular, the parasites of New Guinea wildlife, including hemoparasites, are poorly studied (Austin and Perkins, 2006; Klompen and Austin, 2007). In the course of this survey, we encountered several parasites that were morphologically distinct. However, in each case, very few asexual stages were present; thus, classifying the parasites, even

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* Department of Biological Sciences & Museum of Natural Science, Louisiana State University, 119 Foster Hall, Baton Rouge, Louisiana 70803.

at the generic level, would be problematic if only morphological characters were used. Therefore, we also used sequences from 2 genes and a phylogenetic analysis to determine that these parasites are indeed *Plasmodium*, and that they are distinct enough, particularly when combined with morphological characteristics of the gametocyte stages, to define as new species. The combination of morphological data and morphometrics of gametocytes with sequences of the parasites' cytochrome b (*cytb*) and cytochrome oxidase I (*coxI*) genes from the mitochondrial genome shows 5 monophyletic lineages, 4 of which appear to be new to science (3 from scincid lizard hosts and 1 from an agamid lizard), and a fifth species, which we conservatively place within *Plasmodium lacertiliae*, originally reported on Goodenough Island, off the coast of the main island of New Guinea (Thompson and Hart, 1946).

MATERIALS AND METHODS

Lizards were captured alive by hand or blowpipe. Blood was collected via standard protocols with a heparinized capillary tube from the post-orbital sinus. Thin blood smears on slides were air-dried and then fixed in 95% methanol. All thin blood smears are currently held at the American Museum of Natural History, New York, New York, and hapantotype and parahapantotype specimens have been accessioned into the American Museum of Natural History (AMNH) Protozoan Collection. Blood was also spotted onto filter or FTA paper and air-dried for preservation of DNA and subsequent DNA isolation and amplification. Liver and muscle tissue was taken from vouchers and stored in either 95% ethanol or liquid nitrogen. Lizard host vouchers are deposited at the Louisiana State University Museum of Natural Science, Baton Rouge, Louisiana.

Thin blood smears were stained with Giemsa and examined using a microscope for 3 to 6 min to determine infection status. Slides with parasites were then examined completely, noting various stages present. Measurements of mature gametocytes were performed with a SPOT InTouch® digital camera and the SPOT software version 4.1.3. These metrics included parasite length (as determined by the longest continuous line that could be drawn through a parasite cell), parasite width (the longest line that could be drawn perpendicular to the length line), the length *x* width (LW), and then total parasite and host erythrocyte areas (by tracing the outline of these cells) were measured. The area of the nucleus of a nearby uninfected host cell (= uninfected erythrocyte nucleus, UEN) was also measured in a similar manner for each parasite cell observed. All measurements are reported as means ± standard deviation. Statistical comparisons of morphometrics were performed with 2-tailed unpaired *t*-tests (Welch corrected if there were differences in standard deviations between the populations of measurements) using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, California, www.graphpad.com). Additional observations of parasite morphology, such as shapes of cells, position within the host cell, distortion of the host cell or host nucleus, and presence and distribution of vacuoles and hemozoin pigment, were also noted.

DNA was extracted from either blood spots on filter paper or from host liver tissue. The mitochondrial genes *cytb* and *coxI* were amplified as follows. The *cytb* gene was amplified in 2 fragments, the first with primers DW2 (TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG) and 3932R (GAC CCC AAG GTA ATA CAT AAC CC) and the second with 3932F (GGG TTA TGT ATT ACC TTG GGG TC) and DW4 (TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG). The *coxI* gene was amplified with a nested reaction according to the methods described in Perkins et al. (2007). Briefly, an outer reaction using primers coIF (CTA TTT ATG GTT TTC ATT TTT ATT TGG TA) and coIR (GTA TTT TCT CGT AAT GTT TTA CCA AAG AA) was done, and then this was nested with 2 reactions, 1 with coIinF (ATG ATA TTT ACA RTT CAY GGW ATT ATT ATG) and coImidR (CTG GAT GAC CAA AAA ACC AGA ATA A) and 1 with coImidF (TTA TTC TGG TTT TTT GGT CAT CCA G) and coIinR (GTA TTT TCT CGT AAT GTT TTA CCA AAG AA). PCR products were purified with AMPure (Agencourt, Beverly, Massachusetts) and sequenced in both directions using BigDye v. 3.0 (Applied Biosystems, Foster City, California). Sequences were edited in Sequencher (Gene Codes, Madison, Wisconsin). There was no length variation in the sequences, and alignments were done simply by eye. Several published sequences of other lizard malaria parasite species (Martinsen et al., 2008) were added to the matrix of the newly generated sequences and the mammal-infecting taxa; *Plasmodium berghei* and *Plasmodium vivax* were used as outgroups. The 2 genes were combined, and primer sequences were removed before analysis. Phylogenetic analyses were conducted using maximum likelihood using RaxML (Stamatakis, 2006) via the Cyberinfrastructure for Phylogenetic Research (CIPRES) portal (http://www.phylo.org/news/RAxML), and the data set was partitioned by gene such that separate models of evolution were estimated for each. Bootstrapping (cut-off automatically determined by the software) was used to assess nodal support.

Molecular characters for each of the 4 new species and the redescription of *Plasmodium lacertiliae* were determined by aligning sequences to those published for named species of lizard and bird malaria parasites. These included *Plasmodium mexicanum* (NC_009960), *Plasmodium floridense* (NC_009961), *Plasmodium gallinaceum* (EU254535 and EU254578), *Plasmodium relictum* (AY733090), *Plasmodium azurophilum* (in erythrocytes; EU254532 and EU254575), *Plasmodium azurophilum* (in leucocytes; EU254533 and EU254576), *Plasmodium giganteum* (EU254534 and EU254577), *Plasmodium chiricahuae* (*cytb* only; AY099061), *Plasmodium elongatum* (*cytb* only; AF069611), *Plasmodium agamae* (*cytb* only; AY099048), *Haemoproteus kopki* (*cytb* only; AY099062), and *Haemoproteus ptyodactylii* (*cytb* only; AY099057). Positions refer to those of the complete protein-coding sequence for *Plasmodium falciparum* (NC_002375).

RESULTS

Five distinct morphotypes of parasites were observed in the lizards from Papua New Guinea, and these corresponded exactly with 5 distinct clades or lineages of parasites from the phylogenetic analyses (Fig. 1), each of which was supported with 100% nodal support under either analysis method. One morphotype, observed in 2 *Emoia longicauda* lizards collected in Milne Bay Province, could not be distinguished as different from *P. lacertiliae* Thompson and Hart, 1946, and so it is provisionally identified as this species, though we offer a redescription to update the hosts, localities, and include the DNA sequence data. All sequences are deposited in GenBank under the accession numbers EU834703–EU834720. The *cytb* gene sequence for the isolate from CCA 2054 was identical to that from CCA 2146; likewise, *cytb* sequences from the parasites in CCA 3565 and CCA 3568 were the same as were those sequences from CCA 3597 and CCA 3598. The *coxI* sequences obtained from the malaria parasite infections in CCA 2227 and CCA 2228 were identical.

DESCRIPTIONS

Plasmodium minuoviride n. sp.

(Figs. 2–7)

Asexual stages: Trophozoites small, but often elongated, with large vacuoles, typically at 1 end of cell (Figs. 2, 3). Pigment present in most infected cells. Two host cells observed to have 2 trophozoites each (Figs. 2, 3). Three schizonts observed in single infections; 2 were fan-shaped and appeared to have 4 meronts per cell (e.g., Fig. 4), and 1 was irregularly shaped with 8 meronts (Fig. 5). Asexual stages did not distort host nucleus or cell.

Gametocytes: Gametocytes medium sized, typically round or slightly oval, with 1 or more vacuoles often present (Figs. 6, 7). Vacuoles of gametocytes sometimes smaller than those of trophozoites. Gametocytes located polar in host cell, but did not distort position of host nucleus, or shape of cell. Very small clumps of pigment present in most cells. Gametocyte morphometric characteristics as follows: 10 gametocytes measured, with an average length of 8.06 ± 1.08 (6.32–10.06) μm and an average width of 5.64 ± 1.49 (4.24–8.02) μm; average LW value

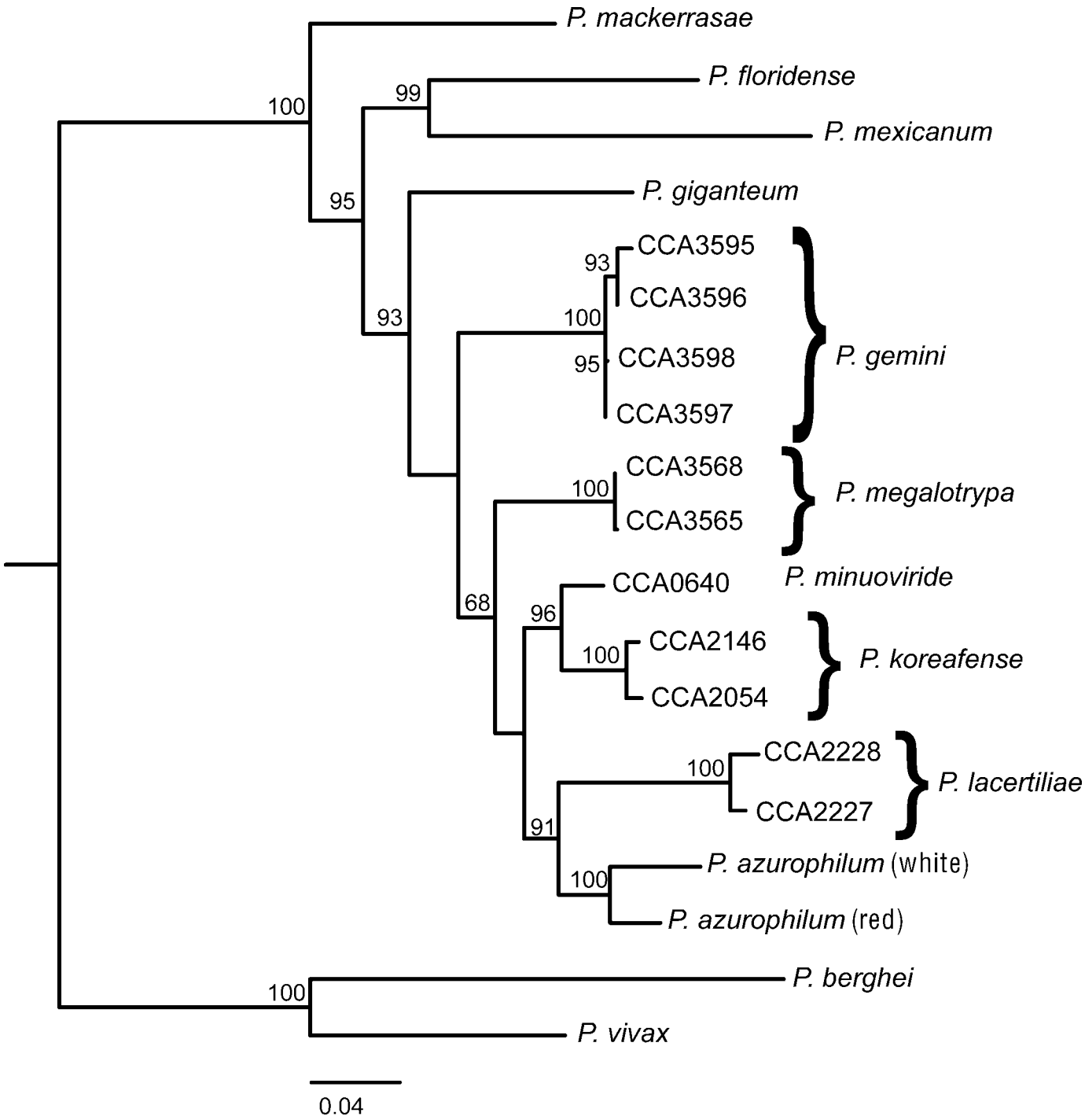


FIGURE 1. Phylogenetic relationships of the 4 newly described species of *Plasmodium* from New Guinea skinks and *P. lacertiliae*, as well as several other lizard malaria parasite taxa, rooted with the mammalian parasites, *P. vivax*, and *P. berghei*. Nodal support values (>50%) from ML analyses are presented above branches.

of these parasites 46.09 ± 15.83 (27.74–74.54) μm^2; size of gametocytes relative to uninfected erythrocyte nuclei 1.51 ± 0.42 (0.97–2.32); proportion of parasite area to total erythrocyte area 0.23 ± 0.08 (0.12–0.36).

Molecular characters: Cytochrome b: No single fixed character differences observed, but combination of T at position 525 with G at position 535 unique to this isolate. Cytochrome oxidase I: No single fixed difference, but combination of C at position 853 and A at position 861 unique to this species.

Taxonomic summary

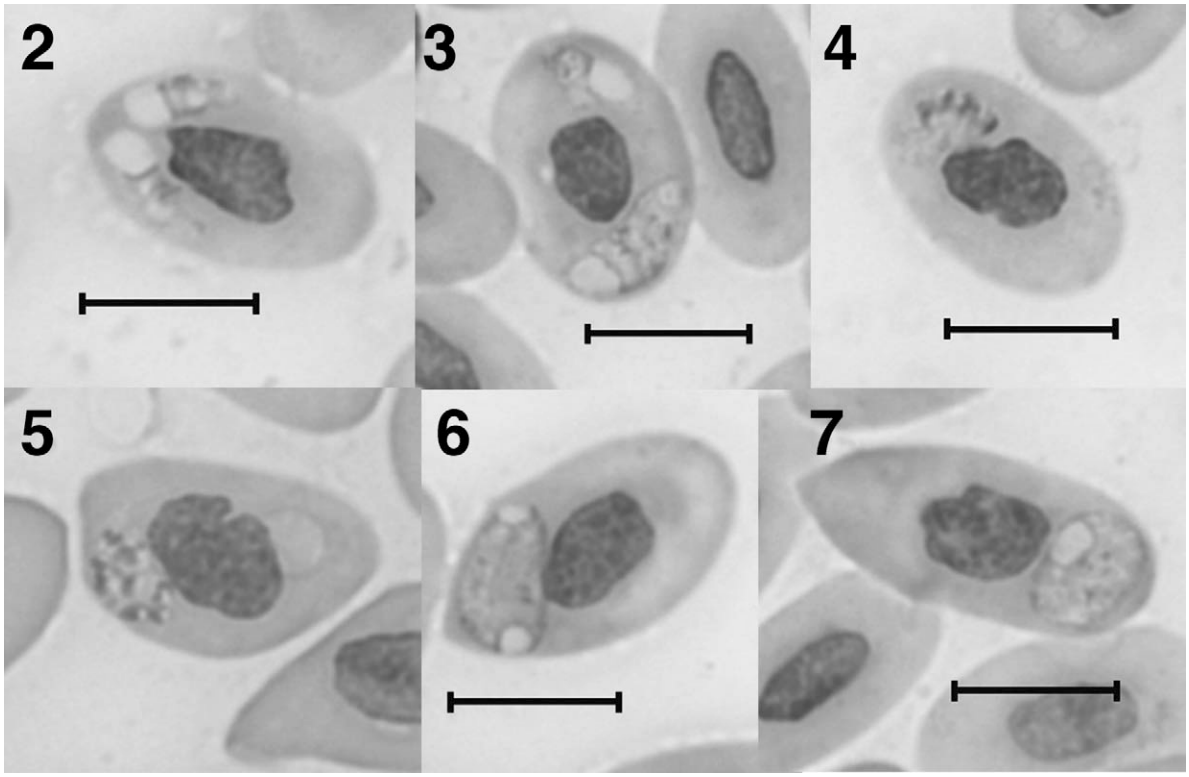
Type host: Prasinohaema prehensicauda (Loveridge, 1945) (Sauria: Scincidae); type host voucher = CCA 0640/ TNHC 51875.

Other hosts: None known.

Type locality: Kaironk Village, Madang Province, Papua New Guinea (05°14′15′′S, 144°28′50′′E, ~1400 m).

Additional localities: None known.

Site of infection: Erythrocytes.



FIGURES 2–7. *P. minuoviride* n. sp. from *Prasinohaema prehensicauda*. Scale bar = 10 μm. (**2–3**) Trophozoites. (**4–5**) Schizonts. (**6–7**) Gametocytes.

Prevalence: One of 23 (7%) *P. prehensicauda* collected at type locality.

Material deposited: AMNH Protozoan Collection #816.

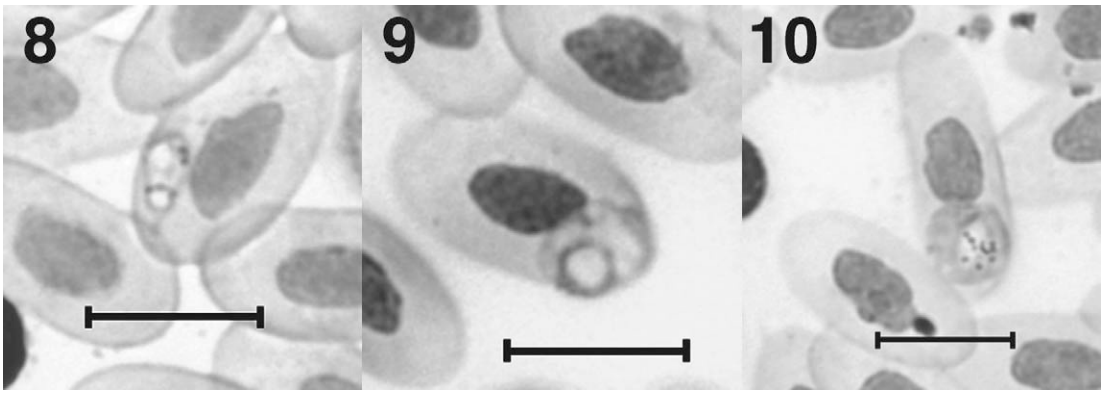
Etymology: The specific name, a combination of the Latin ''*minuo*,'' meaning to draw blood, let blood, or to bleed, and *viride*, meaning green. It refers to this *Plasmodium* species being found in the lizard *P. prehensicauda*, which is characterized by green blood due to accumulation of the bile pigment biliverdin in the tissues and blood (Austin and Jessing, 1994).

***Plasmodium koreafense* n. sp.**
(Figs. 8–10)

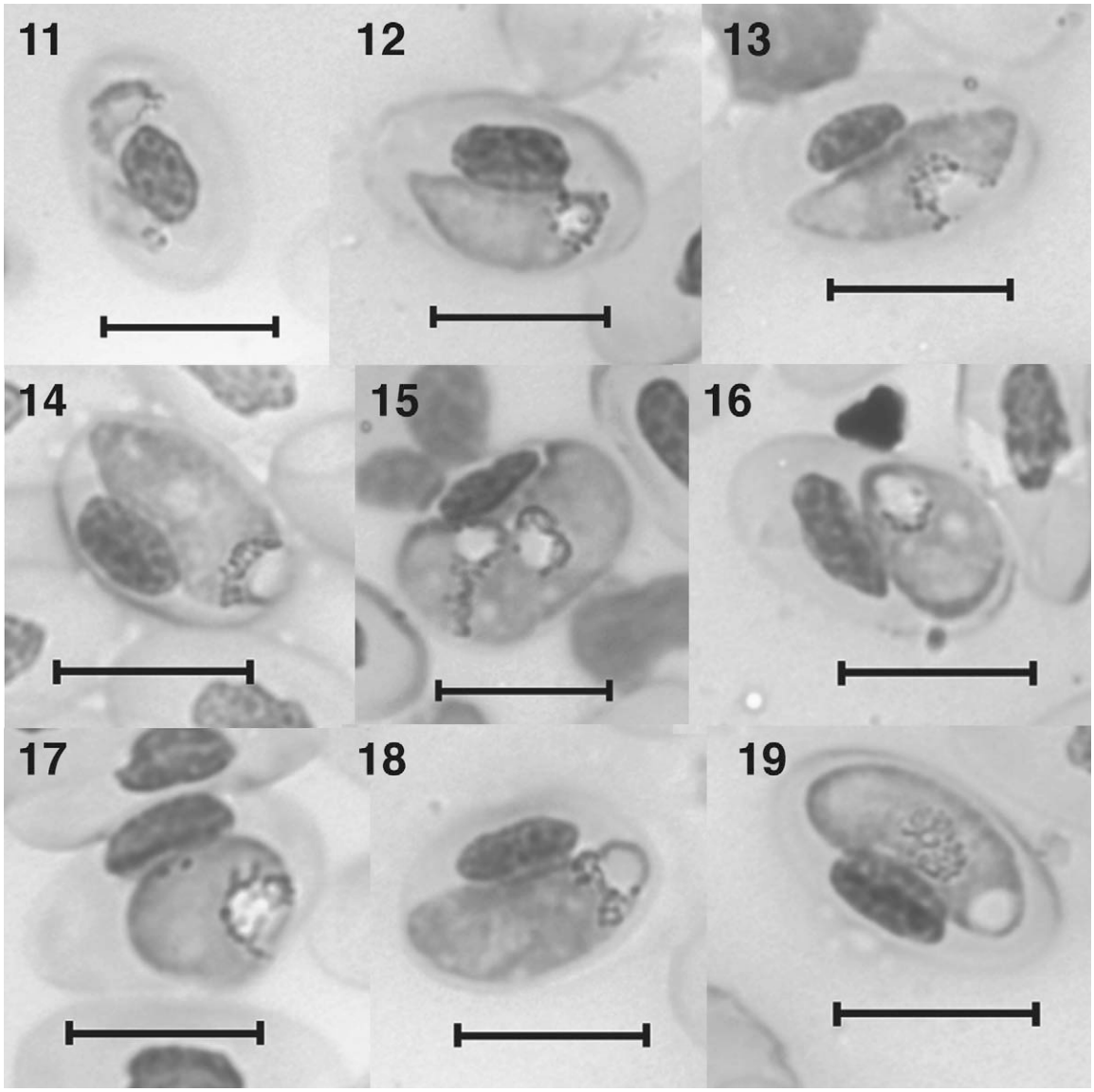
Asexual stages: One possible trophozoite observed (Fig. 8); morphology teardrop-shaped, with prominent vacuole and pigment.

Gametocytes: Gametocytes small to medium sized, typically round and located polar in host cell (Figs. 9, 10); sometimes cause slight longitudinal elongation of host cell (Fig. 10). Very small clumps of pigment present in most cells. Gametocyte morphometric characteristics as follows: 10 gametocytes measured from infection in CCA 2146 with an average length of 6.07 ± 0.60 (5.45–7.20) μm and an average width of 4.97 ± 0.48 (4.22–5.52) μm; average LW value for these parasites 30.22 ± 4.62 (23.08–37.11) μm^2; size of gametocytes relative to uninfected erythrocyte nuclei 1.18 ± 0.27 (0.78–1.62); proportion of parasite area to total erythrocyte area 0.21 ± 0.03 (0.17–0.27). One gametocyte observed in CCA 2054; it appeared to be immature, though consistent with general morphology of CCA 2146.

Molecular characters: Cytochrome *b*: Two isolates of *P. koreafense* showed following 4 unique fixed differences: T at position 156, T at position 321, T at position 342, and C at position 525. Cytochrome oxidase I: Could not obtain full *coxI* sequence from isolate CCA 2054, but CCA 2146 isolate of *P. koreafense* showed 3 unique characters from other taxa: C at position 238, G at position 514, and C at position 789.



FIGURES 8–10. *P. koreafense* n. sp. in *Sphenomorphus jobiensis*. Scale bar = 10 μm. (**8**) Trophozoite. (**9–10**) Gametocytes.



FIGURES 11–19. *P. megalotrypa* n. sp. in *Sphenomorphus simus*. Scale bar = 10 μm. (**11**) Trophozoites. (**12–19**) Gametocytes.

Taxonomic summary

Type host: Sphenomorphus jobiensis (Meyer, 1874) (Sauria: Scincidae); type host voucher = (CCA 2146).

Other hosts: None known.

Type locality: Koreaf Village (09°20.26′S, 149°08.40′E, 30 m), Collingwood Bay, Oro Province, Papua New Guinea.

Additional localities: Tingau Village (02°05.76′S, 147°06.33′E, 296 m), 27 km from Lorengau, Manus Island, Manus Province, Papua New Guinea (= CCA 2054).

Site of infection: Erythrocytes.

Prevalence: One of 1 (100%) of *S. jobiensis* collected at Tingau Village, Manus Island; 1 of 2 (50%) *S. jobiensis* collected at Collingwood Bay, Oro Province.

Material deposited: AMNH Protozoan Collection #817 (hapantotype), #818 (parahapantotype), and #819 (parahapantotype).

Etymology: The specific name refers to the type locality.

***Plasmodium megalotrypa* n. sp.**
(Figs. 11–19)

Asexual stages: Two small trophozoites co-infecting single host cell (Fig. 11).

Gametocytes: Gametocytes medium to large, either elongate or round (Figs. 12–19). Elongate parasite cells sometimes with tapered ends (Figs. 12–14). One or more large vacuoles present; pigment frequently clustered around periphery of vacuole, though not always (cf. Fig. 19). Gametocytes, especially larger ones, displace host cell nucleus and cause expansion of cell in dimension in which they resided. Gametocyte morphometric characteristics as follows: for infection in CCA 3565, 25 gametocytes measured, with an average length of 10.56 ± 1.96 (7.56–13.58) μm and an average width of 4.96 ± 0.80 (3.64–6.47) μm; average LW 53.06 ± 15.40 (30.53–78.17) μm^2; size of gametocytes relative to uninfected erythrocyte nuclei 2.52 ± 0.78 (1.29–4.17); proportion of parasite area to total erythrocyte area 0.34 ± 0.10 (0.19–0.55). Twenty-five gametocytes measured for CCA 3568: average length 10.16 ± 1.64 (7.57–12.74) μm, and average width 5.39 ± 0.94 (3.66–7.24) μm; average LW product 54.20 ± 10.64 (37.76–75.68) μm^2. Ratio of parasite area to that of nearby uninfected erythrocyte nuclei 2.49 ± 0.58 (1.57–3.78); proportion of area of host cell encompassed by parasite 0.38 ± 0.06 (0.23–0.50); *t*-tests of 2 infections indicate no significant differences in measurements (length × width: parasite area, $P = 0.7653$; uninfected erythrocyte area, $P = 0.8797$; proportion of total cell area occupied by parasite, $P = 0.0810$).

Molecular characters: Cytochrome b: Two isolates of *P. megalotrypa*

with following 5 unique fixed differences: C at position 210, G at position 243, C at position 289, C at position 510, and C at position 1068. Cytochrome oxidase I: Eight unique differences in *coxI* gene for *P. megalotrypa*: C at position 292, C at position 774, C at position 786, G at position 796, A at position 936, G at position 937, G at position 1106, and C at position 1119.

Taxonomic summary

Type host: Sphenomorphus simus (Sauvage, 1879) (Sauria: Scincidae); type host vouchers = CCA 3565.

Other hosts: Also found in a second *S. simus* (CCA 3568) at type locality.

Type locality: Bewani Station (03°3.224′S, 141°10.009′E, 181 m), Sandaun Province, Papua New Guinea.

Additional localities: None known.

Site of infection: Erythrocytes.

Prevalence: Two of 5 (40%) *S. simus* collected at Bewani Station.

Material deposited: AMNH Protozoan Collection #820 (hapantotype) and #821 (parahapantotype).

Etymology: The specific name, a noun in apposition, refers to the large vacuoles present in the gametocyte stages (Greek for ''big hole'').

Plasmodium gemini n. sp.

(Figs. 20–31)

Asexual stages: Small, round, or amoebic trophozoites observed in 2 infected lizards (Figs. 20, 21). Small vacuoles and tiny bits of pigment present.

Gametocytes: Gametocytes either nearly round and polar (Fig. 23), or long and elongate (Figs. 24–31). If second kind, often found in pairs (Figs. 27–29; approximately 20% of all infected cells) or (in a single instance) 4 in single erythrocyte (Fig. 30). Both macrogametocytes and microgametocytes sometimes in same erythrocyte (Figs. 28, 31). Twice, gametocytes observed with unusual morphology of elongate cell and unusual bulge in center (Fig. 31). For infection in CCA 3595, 41 gametocytes measured, with average length of 8.43 ± 1.72 (4.83–11.66) μm and average width of 3.93 ± 0.61 (2.71–4.84) μm; average LW of 32.68 ± 6.72 (19.06–48.60) μm^2. Size of gametocytes relative to uninfected erythrocyte nuclei 1.02 ± 0.24 (0.54–1.46) and proportion of parasite area to total erythrocyte area 0.21 ± 0.05 (0.12–0.31). Five gametocytes measured for CCA 3596: average length 5.58 ± 0.96 (4.83–7.24) μm, average width 4.36 ± 0.53 (3.55–4.85) μm, and average LW 24.20 ± 4.42 (19.24–30.84) μm^2. Ratio of parasite area to nearby uninfected erythrocyte nuclei 0.76 ± 0.14 (0.65–0.99) and proportion of area of host cell encompassed by parasite 0.17 ± 0.04 (0.12–0.21). Two of 3 metrics significantly different when compared via *t*-tests ($P = 0.009$; $P = 0.01$; $P = 0.10$); however, since only round gametocytes were observed in CCA 3596, when compared to only round gametocytes of CCA3595, differences for 3 measures were not significant ($P = 0.52$; $P = 0.79$; $P = 0.86$). It is still believed that CCA3595 represents a single infection; there is no evidence of multiple peaks in sequences of 2 genes for that infection because all came from same host species at same locale. Despite different shape, both parasites types are similar in lacking pigment and vacuoles.

Molecular characters: Cytochrome b: Four isolates of *P. gemini* with 2 unique fixed differences: T at position 141 and A at position 549. Cytochrome oxidase I: Eight fixed differences unique to 4 isolates of *P. gemini*: G at position 249, T at position 276, G at position 291, A at position 448, C at position 865, A at position 874, C at position 1048, and C at position 1089.

Taxonomic summary

Type host: Hypsilurus modestus Meyer 1874 (Sauria: Agamidae); type host voucher = CCA 3595.

Other hosts: Found in 3 other *H. modestus* at same locality (vouchers CCA 3596, CCA 3597, and CCA 3598).

Type locality: Utai Village (3°23.765′S, 141°34.974′E, 208 m) in Sandaun Province, north-central Papua New Guinea.

Additional localities: None known.

Site of infection: Erythrocytes.

Prevalence: Four of 6 (67%).

Material deposited: AMNH Protozoan Collection #822 (hapantotype) and #823–825 (parahapantotypes)

Etymology: The specific name refers to the propensity of this parasite to be found ''twinned'' in host cells.

REDESCRIPTION

Plasmodium lacertiliae Thompson and Hart, 1946

(Figs. 32–37)

Asexual stages: One RBC (Fig. 32) infected with 2 ring-shaped trophozoites. One schizont observed in apparent lizard thrombocyte; irregularly shaped, with 9 visible merozoites (Fig. 33).

Gametocytes: Gametocytes medium to large, typically elongated, lying alongside host nucleus in either longitudinal direction (Figs. 34–36; 92% of those observed), or partially polar (Fig. 37; 8%). Occasionally, parasite cell wrapped around both ends of nucleus (Figs. 36, 37). No vacuoles observed. Gametocytes sometimes distort position of host nucleus (Fig. 34), but not shape of RBC. Pigment present in most cells, often scattered throughout parasite cell. Gametocyte morphometric characteristics as follows: For infection in CCA 2227, 19 gametocytes measured, with average length of 14.09 ± 0.43 (10.25–16.72) μm and average width of 5.69 ± 0.18 (3.89–7.62) μm. Length × width for these parasites average 80.45 ± 2.91 (53.68–111.56). Size of gametocytes relative to uninfected erythrocyte nuclei 3.65 ± 0.19 (2.08–5.01) and proportion of parasite area to total cell area 0.61 ± 0.02 (0.39–0.78). In total, 25 gametocytes measured for CCA 2228. Average length 13.67 ± 0.36 (8.97–17.51) μm; average width 5.43 ± 0.22 (3.96–7.42) μm; average product 73.61 ± 3.20 (57.54–114.52). Ratio of parasite area to that of nearby uninfected erythrocyte nuclei 4.05 ± 0.21 (2.46–6.92) and proportion of area of host cell encompassed by parasite 0.56 ± 0.02 (0.37–0.71); *t*-tests showed no significant differences in measurements (length × width, $P = 0.1733$; parasite area: UEN, $P = 0.1678$; proportion of total cell area occupied by parasite, $P = 0.0852$).

Molecular characters: Cytochrome b: Both CCA 2227 and CCA 2228 with unique difference in C at position 309, and, though complete sequence unavailable for CCA 2227, CCA 2228 unique at position 555 in having C. Cytochrome oxidase I: The 2 isolates identified as *P. lacertiliae* showed 11 unique fixed differences: C at position 444, G at position G, A at position 633, C at position 669, T at position 750, T at position 799, G at position 832, A at position 840, C at position 864, C at position 1101, and C at position 1186.

Taxonomic summary

Host: Emoia longicauda (MacLeay, 1877) (Sauria: Scincidae). Host vouchers = CCA 2227 and CCA 2228.

Other hosts: Leiolopisma fuscum, now *Carlia eothen* (type host).

Type locality: Goodenough Island, (09°17.910′S, 150°18.261′E) Papua New Guinea.

Additional localities: Lelegalu Village (10°19.40′S, 150°41.56′E, 5 m), East Cape, Milne Bay Province, Papua New Guinea.

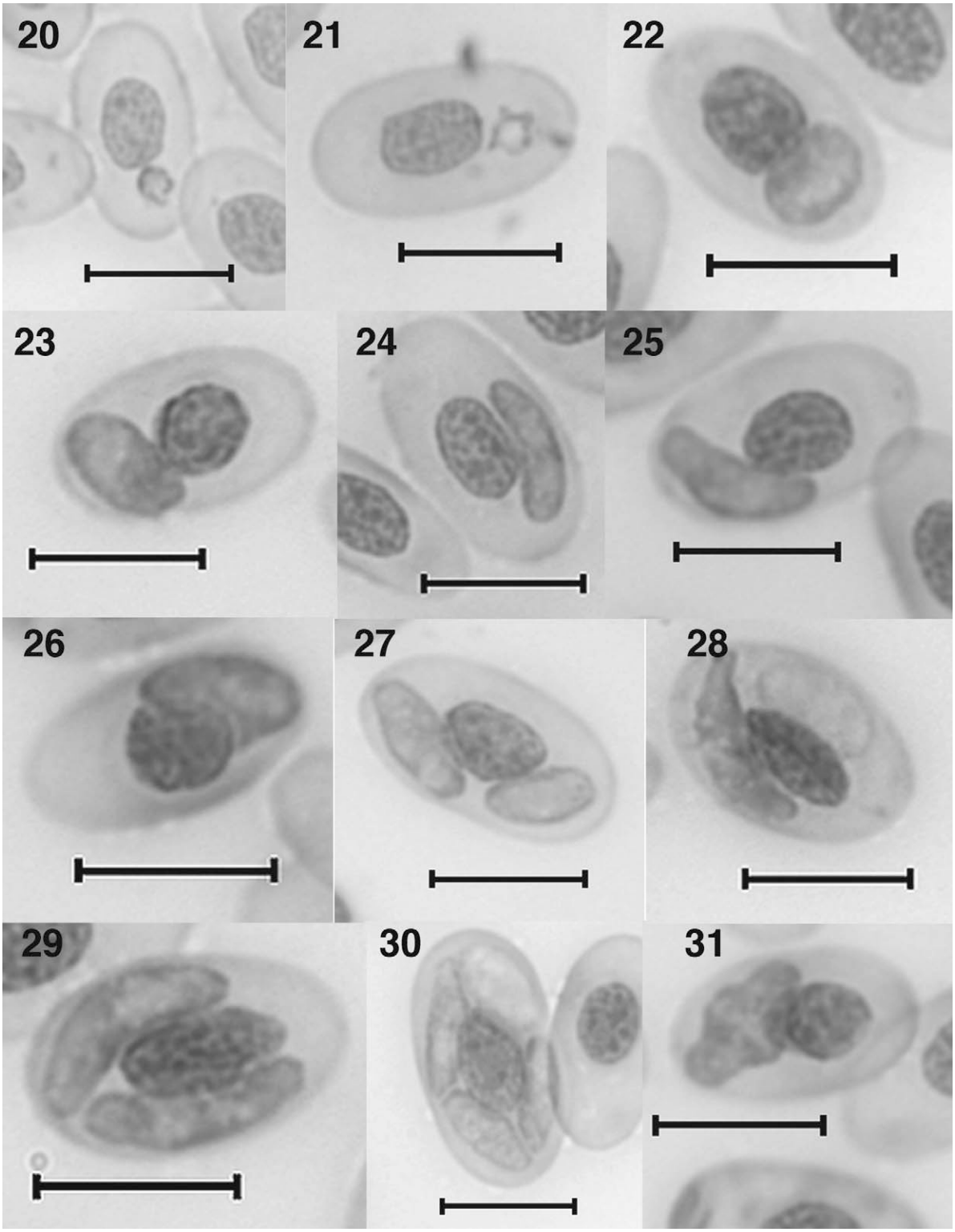
Site of infection: Erythrocytes.

Prevalence: Two of 2 (100%) *E. longicauda* collected at Lelegalu Village. Another specimen of this host species collected at Oro was not infected.

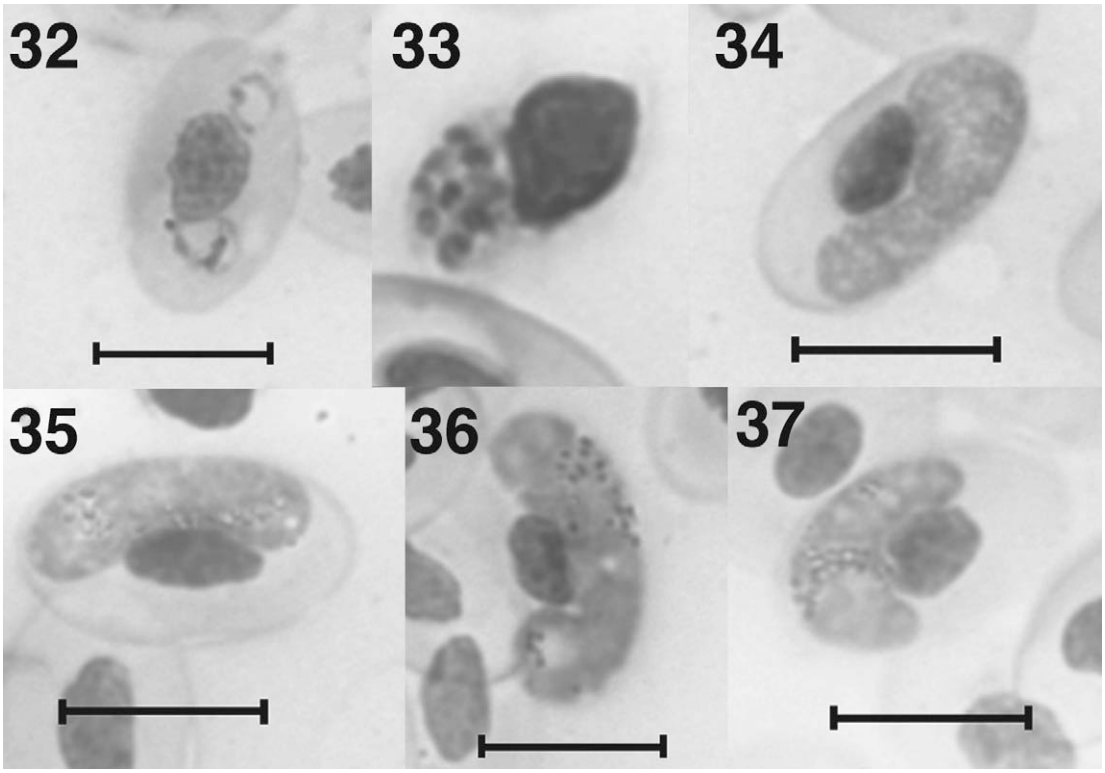
Material deposited: AMNH Protozoan Collection #826 and #827.

REMARKS

To date, just 2 species of *Plasmodium* in lizards believed to have originated from the main island of New Guinea have been described, i.e., *Plasmodium tribolonoti* and *Plasmodium gracilis*, which were found infecting a single *Tribolonotus gracilis*, a skink, from the pet trade that was reasoned to have come from Indonesian New Guinea (Telford and Wellehan, 2005). The only new species described herein that resembles these 2 species is *P. megalotrypa*, and it can be distinguished from both of these in that the latter possesses vacuoles in both trophozoite and gametocyte stages. The 2 infections in *Emoia longicauda* were inferred to be *P. lacertiliae*, due to similarities in parasite morphology (only average length and width values are provided in the original species description, but they do fall within the



FIGURES 20–31. *P. gemini* in *Hypsilurus modestus*. Scale bar = 10 μm. (**20–21**) Trophozoites. (**22–31**) Gametocytes. (**27–29**) ''Twinned'' gametocytes in an erythrocyte. (**30**) Quartet of gametocytes: 3 macrogametocytes and 1 microgametocyte in an erythrocyte. (**31**) Unusual ''bulge'' morphology in gametocyte.



FIGURES 32–37. *P. lacertiliae* in *Emoia longicauda*. Scale bar = 10 μm. (**32**) Trophozoites. (**33**) Schizont. (**34–37**) Gametocytes.

ranges for these values observed in our *E. longicauda* parasites) and the geographic proximity of Goodenough Island to Milne Bay Province, where these skinks were captured. This would, however, be the only instance thus far observed in New Guinea of a parasite species infecting multiple host species, let alone multiple host genera; thus, further research may show the *Plasmodium* species infecting *E. longicauda* to be distinct. Some similarities exist between previously described *Plasmodium* species and the novel ones reported herein; however, we feel confident that the latter species are unique. For example, *Plasmodium lygosomae*, a parasite from New Zealand skinks, is similar to *P. megalotrypa*; however, the 2 can be distinguished by the size of vacuoles and the position of pigment in the mature gametocytes. The pigment granuoles in *P. megalotrypa* are characteristically often clumped around the periphery of the large vacuoles in this species, as opposed to the scattered pigment and small vacuoles of *P. lygosomae*. *Plasmodium mackerrasae* overlaps with many of these species in terms of size and shape; however, none of the unusual phaenerozoites observed by Telford and Stein (2000) in *P. mackerrasae* was seen in any infection from Papua New Guinea, and genetic data show that *P. mackerrasae* is not closely related to any of the PNG parasites (Fig. 1). Two of the new species described herein, *P. koreafense* and *P. megalotrypa*, are similar in overall shape, but they differ in size; *P. megalotrypa* is significantly longer ($P < 0.0001$) and possesses a significantly larger area ($P < 0.0001$). *Plasmodium minuoviride* and *P. koreafense* are similar in morphology as well, but the latter does not have vacuoles in the gametocytes.

Our molecular analyses, though based on a very limited number of samples and spotty geographic sampling, show several intriguing phylogenetic and biogeographic patterns. First, the parasites identified as *P. lacertiliae* are sister to the 2 lineages of *P. azurophilum* that infect erythrocytes and leucocytes (Perkins, 2000), despite the fact that these species infect *Anolis* spp. lizards of the Caribbean and have a very different morphology, i.e., rounded gametocytes and no pigment ever sequestered in their cells. Second, the biogeographically most proximate species, *P. mackerrasae* from Australia, is phylogenetically quite distant from the New Guinea *Plasmodium* species radiation and is sister to all of the lizard malaria parasites in the data set. Third, the *Plasmodium* species infecting New Guinea lizards are not a monophyletic group, and, although there is strong host specificity, the parasite phylogeny does not mirror what is known about host relationships (C. Austin, pers. obs.).

The use of DNA sequences for the identification and description of malaria parasites and other parasites with complex life cycles will open up new doors for documenting the biodiversity present in regions and communities and allow for better sets of baseline data with which to identify host switching events or other phenomena important in wildlife and human disease transmission. Although DNA bar coding and DNA taxonomy have clearly sparked a great deal of debate, as Besansky et al. (2003) sagely pointed out, ''The role of any molecular diagnostic is to aid research, not to serve as an end in itself.'' Thus, the molecular characters provided here should be interpreted as a hypothesis of taxonomic status for now; obviously, as new taxa are added to the data matrix, it is possible that some of these fixed characters may, in fact, turn out not to be fixed, but the iterative process of observing parasites and determining DNA sequences will facilitate continued understanding of the taxonomy, systematics, and biogeography of these parasites.

However, we also argue that elements of traditional taxonomy must still be included in descriptions and phylogenetic studies for several reasons. First, if morphological attributes of the parasites are ignored, this will eliminate the possibilities for the study of their evolutionary patterns, e.g., the evolution of their ability to make and store hemozoin pigment (Martinsen et al., 2008) or the propensity to infect certain types of blood cells (Perkins, 2000). These patterns can contribute valuable knowledge about the biology of malaria parasites and could even hopefully be used via comparative methods and genomic approaches to target receptors, metabolic genes, or other pathways that could be used to better understand the organisms' relationship to disease. Second, even the most rudimentary examination of morphological characteristics of the parasites can alert one to potential sources of contamination or multiple infections. Finally, the skills of traditional parasite taxonomists should not be under-rated in terms of speed and reliability of identifying parasite species, including both species that have been previously described and those that might be new to science. Although distillation of the morphology of a simple protozoan cell into a series of lengths, widths, and area measurements cannot always allow for complete discrimination of species, an experienced malariologist can nonetheless often reliably and quickly identify parasites within a blood smear when familiar with their basic morphology. Cataloging the biodiversity of organisms on the planet is ever more urgent. Discovering and identifying parasites is also pressing given their important roles in conservation, species richness, and also because of potential zoonoses that are increasing in frequency as natural habitats shrink and change. At the same time, largely because of the increase in availability of molecular markers, new studies are being published that incorporate novel lineages of parasites but that are either not identified to species or described as new species but are left simply as ''*Plasmodium* sp.'' in publications and public data records. This creates a problem because there are no host vouchers or very rarely even any specimens of these parasites deposited into collections that can be used for comparison by other researchers. This trend has recently been discussed and admonished in the parasitological literature (Valkiunas et al., 2008). Thus, we are currently in a very precarious situation. Should potential new species of malaria parasites then be shelved until additional samples are collected that contain all life-history stages? This seems a taxonomic waste, particularly in light of the very real possibility that their hosts might soon go extinct in many of these places, taking these parasite species with them forever. Should we continue to leave them as multitudes of identically labeled ''*Plasmodium* sp.'' in any studies of the molecular systematics and evolution of these organisms? This practice will inevitably contribute to confusion and misuse. Instead, we propose a compromise, i.e., integrated taxonomy involving the coupled evidence of, in this case, morphological data from as many parasite life-history stages as possible (recognizing that not all might be currently available) and DNA sequences. We recognize that this compromise does not perfectly match the ideal guidelines of alpha taxonomy as outlined by Dayrat (2005), primarily because the genus has not recently been monographed and because parasite prevalence and parasitemia are often low in natural populations, making it difficult to thoroughly explore the range of intra- and interspecific variation. However, in cases such as the ones described herein, where morphological and molecular differences (and in almost all, host preferences, as well) clearly differentiate lineages, where parasites are distinguishable from any geographically proximate taxa, and where conservative identifications are made and species redescribed as opposed to created, until further evidence can be obtained, we believe that this scenario is the lesser of 2 evils and within the bounds of solid taxonomic practice. Most emphatically, we second the plea from Dayrat (2005) and Valkiunas et al. (2008) that specimens should be deposited into museum collections and further this by encouraging that host tissues or other samples that could be used for future molecular work also be made available or at least traceable to other researchers. Should new specimens be collected that contain additional life-history stages of parasites, these can then easily be compared to the hapantotype and parahapantotype (Williams, 1986) vouchers, and the taxonomy can either be updated, or, if need be, revised. Taxonomy is, after all, a dynamic science, and both species and their phylogenetic relationships are merely the currently best-supported hypotheses.

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