

## LARGE SEQUENCE HETEROGENEITY OF THE SMALL SUBUNIT RIBOSOMAL RNA GENE OF *PLASMODIUM OVALE* IN CAMBODIA

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**Abstract.** *Plasmodium ovale* malaria has been reported in various countries in southeast Asia, but never in Cambodia. Using a species-specific polymerase chain reaction (PCR) targeting the small subunit (SSU) ribosomal RNA (rRNA) gene, we detected *P. ovale* in nearly 4% of the inhabitants of a northeastern Cambodian village. *Plasmodium ovale* was associated with at least one other *Plasmodium* species, and two quadruple infections were detected. The diagnosis was confirmed by microscopy and by SSU rRNA PCR product sequencing. The sequences shared 96–99% identity with published sequences, and displayed a substantial heterogeneity with 2–4 different haplotypes per sample. Nine distinct SSU rRNA haplotypes were identified, including seven novel variants. Phylogenetic analysis showed two major genetic clusters, suggesting amplification of two distinct gene sets and/or *P. ovale* variants from each sample. Our data indicate that *P. ovale* was overlooked in Cambodia until now, and call for the implementation of larger prevalence surveys and accurate diagnosis methods in this country.

### INTRODUCTION

The incidence of malaria has decreased considerably over recent decades in most countries in the Mekong sub-region. However, the transmission rate remains unacceptably high in several areas, including along the Cambodian borders with Thailand, Laos, and Vietnam.<sup>1,2</sup> *Plasmodium falciparum* and *P. vivax* infections are predominant in Cambodia. In 2003, they accounted for 86% and 12% of the malaria cases reported to the National Center for Parasitology, Entomology and Malaria Control (Phnom Penh, Cambodia), respectively.<sup>3</sup> The prevalence of *P. malariae* is unclear. Infections with *P. malariae* were detected in large numbers in the 1950s and 1960s,<sup>4,5</sup> but have become rare in the past 20 years and are not systematically recorded and notified by the Cambodian health services.

*Plasmodium ovale* was long thought to be absent from southeast Asia. The first microscopic identifications of *P. ovale* were reported in the 1970s in countries surrounding Cambodia,<sup>6–9</sup> including cases along the eastern and northwestern borders of Cambodia.<sup>10–12</sup> Molecular diagnosis methods have recently led to the identification of even more *P. malariae* and *P. ovale* infections in the southeast Asian region.<sup>13–18</sup> However, until now, *P. ovale* malaria has never been diagnosed in Cambodia.

We used a highly sensitive polymerase chain reaction (PCR)-based method based on the amplification of the small subunit (SSU) ribosomal RNA (rRNA) gene in conjunction with microscopy to study the prevalence of the four human malaria species in blood specimens from inhabitants of the Rattanakiri province of Cambodia (Incardona S and others, unpublished data). This led to the identification of *P. ovale* malaria cases in the area. The PCR products amplified from these *P. ovale* specimens were also cloned and sequenced. This showed a surprisingly high SSU rRNA gene sequence heterogeneity between different isolates and between different clones within each isolate, and novel *P. ovale* variants were detected. Previously published *P. ovale* SSU rRNA sequences are generally of high similarity, but some extent of variation has been reported for a small number of cases. The complete gene sequence of a Cameroonian isolate differs by

4% from other African and Asian isolates.<sup>19</sup> One *P. ovale* variant was observed in several places in the southeast Asian region.<sup>13,15,18</sup> More recent data with detailed molecular analysis of a small number of *P. ovale* isolates show sequence variations in the SSU rRNA gene as well, and the existence of *P. ovale* subpopulations with two major variant types (called the CDC type and the LS type) has been suggested.<sup>20,21</sup> Nevertheless, compared with *P. falciparum* and *P. vivax*, few data about the SSU rRNA genes of *P. ovale* are available. The results described in this report show that large sequence heterogeneity can exist in the SSU rRNA gene of wild *P. ovale* isolates, even within a very limited geographic area, and within individual *P. ovale* isolates. This diversity can have consequences on the molecular species diagnosis using *P. ovale*-specific primers, but our findings also raise new questions regarding the clonality of *P. ovale* infections, and the eventual existence of polymorphic and/or stage-specific SSU rRNA gene copies.

### MATERIALS AND METHODS

**Sample site and blood collection.** Blood samples were collected from all 102 available inhabitants of Ping, a village in the Rattanakiri Province, during a peak season cross-sectional prevalence survey in September 2001. Rattanakiri is located in northeastern Cambodia, close to the borders with Laos and Vietnam, and is home to most of Cambodia's ethnic minority groups. It is characterized by a high malaria transmission rate and low levels of drug resistance compared with the rest of the country. The estimated annual parasite incidence in this province was 26.3 per 1,000 inhabitants in 2003, with 82% *P. falciparum*-, 17.7% *P. vivax*-, and 0.3% *P. falciparum/P. vivax* mixed infections.<sup>3</sup> Ping had the highest prevalence of the 38 villages included in this survey, with nearly 60% *Plasmodium* carriers identified by microscopy, and a *P. falciparum* prevalence reaching 81% in children 2–9 years old.

For each enrolled individual, personal and clinical information was recorded, a finger prick blood sample was obtained, and thin and thick blood smears were prepared. Blood spots

of approximately 25  $\mu\text{L}$  were also collected on Whatman (Brentford, United Kingdom) 3M filter paper. The Giemsa-stained blood smears were examined at the National Center for Parasitology, Entomology and Malaria Control in Phnom Penh. The blood spots were analyzed by the Pasteur Institute of Cambodia.

This study was reviewed and approved by the National Ethics Committee of the Kingdom of Cambodia, and all participants gave informed consent.

**Extraction of DNA and characterization of SSU rRNA sequences.** Parasite DNA was extracted from dried blood spots in a 96-well plate format by using the QIAamp DNA blood 96 kit in combination with Qiagen ATL buffer (Qiagen, Hilden, Germany), using a slightly modified version of a previously described protocol.<sup>22</sup> A nested PCR of the SSU rRNA genes was performed essentially as described.<sup>23</sup> Briefly, 4  $\mu\text{L}$  of DNA was amplified in a 50- $\mu\text{L}$  reaction volume with the genus-specific primers rPLU1 and rPLU5. The cycling conditions were as follows: 94°C for 4 minutes, then 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 4 minutes. A *Plasmodium*-specific nested PCR was performed with the primers rPLU3 and rPLU4 and 2  $\mu\text{L}$  of PCR product. The cycling conditions were the same as above, except that the annealing temperature was 62°C. For all samples that were positive, a species-specific nested PCR was performed in four separate 20- $\mu\text{L}$  reactions with the four primer pairs rFAL1/rFAL2, rVIV1/rVIV2, rMAL1/rMAL2, and rOVA1/rOVA2, 2  $\mu\text{L}$  of the PCR product and an annealing temperature of 58°C. The nested PCR products were analyzed by agarose gel electrophoresis and staining with ethidium bromide. The results were interpreted without prior knowledge of the microscopy results. The species-specific nested PCR products were purified with the QIAquick PCR purification kit (Qiagen). The *P. ovale*-specific nested PCR products were cloned using the pGEM-T Vector System I kit (Promega, Madison WI). Double-strand sequencing was performed by Genome Express (Meylan, France) by using the *P. ovale*-specific forward primer rOVA1 and the *Plasmodium*-specific reverse primer 2 described by Kimura and others.<sup>24</sup> Resulting sequences were aligned with previously published sequences from the GenBank/EMBL/DDBJ database, using the CLUSTAL-W alignment program.<sup>25</sup> Phylogenetic analysis was performed with the PHYLIP package version 3.6a3,<sup>26</sup> using the DNAdist algorithm and the neighbor-joining method. The reliability of the tree was assessed by the bootstrap method with 1,000 replications.

## RESULTS

We examined 102 blood specimens from the inhabitants of Ping in the Rattanakiri Province by nested PCR amplification of the SSU rRNA gene, and 75 samples were found to be positive for *Plasmodium* (Incardona S and others, unpublished data). Four of these samples were *P. ovale* positive, giving a prevalence of 3.9% *P. ovale* carriers in the general population. *Plasmodium ovale* was always found in combination with other *Plasmodium* species. One double (*P. falciparum* and *P. ovale*), one triple (*P. falciparum*, *P. vivax*, and *P. ovale*), and two quadruple infections (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) were detected (Figure 1A).

Three of the four *P. ovale*-positive individuals had no clinical symptoms at the time of blood collection (subjects 14, 47, and 94). No clinical information was available for subject 60. Consistent with the molecular approach being the most sensitive method for the detection of minor species, only *P. falciparum* and *P. vivax* parasites were identified by microscopy upon initial examination. Based on the nested PCR results, the microscopy slides were carefully re-examined by an experienced microscopist. In two cases, the parasites meeting the criteria for *P. ovale* at different developmental stages were observed: young trophozoites in ovoid erythrocytes with Schüffner's dots, schizonts with moderate numbers of merozoites in slightly enlarged erythrocytes, and round gametocytes with coarse scattered pigment (Figure 1B). However, because of extremely low parasite densities, with no more than six morphologically confirmed *P. ovale* parasites per slide, slides had to be carefully examined for at least 30 minutes before *P. ovale* parasites were unambiguously identified.

To confirm the presence of *P. ovale* and other species in the four mixed infections, the PCR fragments obtained by species-specific amplification of the SSU rRNA gene were purified and sequenced. The sequences of the *P. falciparum*-, *P. vivax*-, and *P. malariae*-specific PCR products were strictly identical to previously published SSU rRNA sequences of these species (accession numbers M19173, U07367, and M54897, respectively). The partial sequences of the SSU rRNA gene, amplified from the Cambodian DNA samples with *P. ovale*-specific primers, displayed 96–99% identity with previously reported *P. ovale* SSU rRNA sequences (accession numbers L48987, L48986, AF145337, and AJ001527), confirming their species identification.

Three of the four *P. ovale* sequences (#14, #47, and #94) contained ambiguous signals at various positions of the SSU rRNA gene sequence (positions 853, 1134, and 1135; Figure 2). The corresponding PCR fragments were therefore cloned, and four or five randomly selected clones per sample (clones A to E) were sequenced. Consistent with distinct SSU rRNA sequences previously co-amplified by PCR, two to four different haplotypes were identified for each PCR product, i.e., for each isolate. Comparison of the various *P. ovale* SSU rRNA sequences showed further sequence diversity, with a total of nine different haplotypes. The previously published *P. ovale* sequences CDC1 and CDC2 (accession numbers L48987 and L48986) were detected in 33% (5 of 15) of the Cambodian sequences (clones Cb 14A, B, D, and Cb 94A, E), whereas all other haplotypes corresponded to new variants of the *P. ovale* SSU rRNA gene. These new variants resembled either the CDC1 or the CDC2 sequences, with minor variations or combinations of the two haplotypes. In particular, the C to G mutation detected at position 1134 in four different clones (clones Cb 14C, Cb 47C and Cb 94B, D) had never been previously reported.

The *P. ovale* SSU rRNA sequences were also aligned with the stage-specific A, S, and O type SSU rRNA sequences of *P. vivax* (accession numbers U07367, U07368, and U93095, respectively). Among the 10 polymorphic positions analyzed in our study, four positions located in the V5 variable region of the SSU rRNA gene (1112, 1134, 1135, and 1149) appeared to be interesting: the corresponding bases of the *P. vivax* A-type (GGAA) and O-type (AAGG) sequences followed the same pattern as those of the *P. ovale* "CDC1-like" and "CDC2-like" sequences, respectively (Figure 2). The pattern

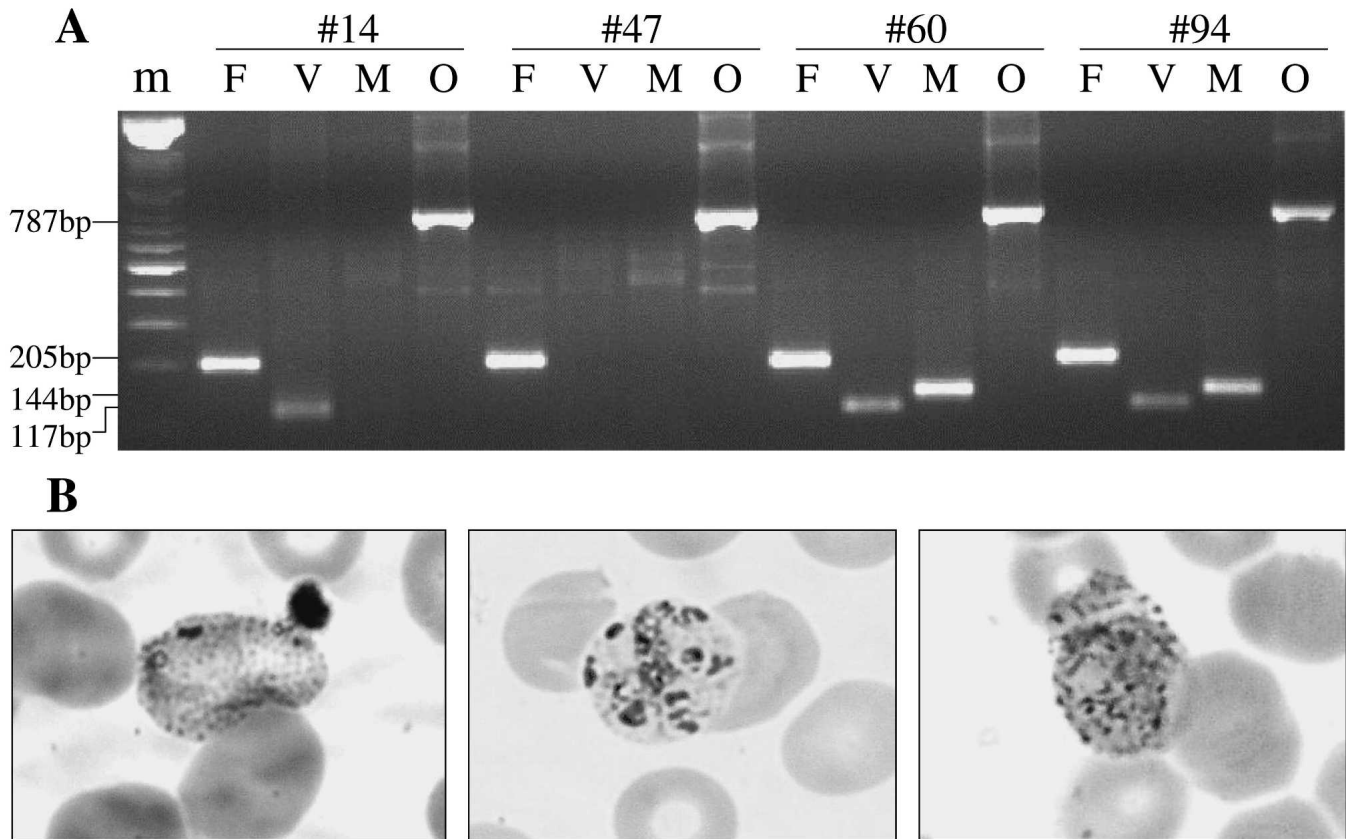


FIGURE 1. **A**, Diagnosis of *Plasmodium ovale* by a nested polymerase chain reaction (PCR). The DNA was extracted from blood spots and amplified by a *Plasmodium* species-specific nested PCR. The PCR products were subjected to electrophoresis on agarose gels and stained with ethidium bromide. Four mixed infections including *P. ovale* were identified: sample #14 (FVO), #47 (FO), #60 (FVMO), and #94 (FVMO). m = DNA molecular mass Marker XIV (Roche Diagnostics, Meylan, France); F = *P. falciparum* (205 basepairs [bp]); V = *P. vivax* (117 bp); M = *P. malariae* (144 bp); O = *P. ovale* (787 bp). **B**, Confirmation of *P. ovale* infection by microscopy. Thin smears were prepared from a finger prick blood sample, fixed in methanol, and stained with 3% Giemsa for 30 minutes. *Plasmodium ovale*-infected erythrocytes were identified on the slides from subjects #14 and #60. The figure shows a young trophozoite of round and regular shape in an ovoid and slightly distorted red blood cell (**left**), a schizont in a slightly enlarged red blood cell and containing 10 merozoites (**middle**), and a round gametocyte with coarse scattered pigment (**right**). (Original magnification  $\times 1,000$ .)

of the *P. vivax* S-type (GGTA) was nearly the same as the CDC1-like haplotypes.

A phylogenetic tree was generated based on the alignment of the *P. ovale* SSU rRNA sequences, and its reliability was tested by the bootstrap method. Consistent with the two types of sequences observed in the alignment, the *P. ovale* SSU rRNA sequences fell into two major genetic clusters, with a reliability of 94.1% (Figure 3). Indeed, the two clones (CDC1 and CDC2) derived from the Nigerian I/CDC strain, as well as the SSU rRNA sequences obtained from the Cambodian samples #47 and #94, fell into these two genetic groups. In contrast, all SSU rRNA sequences obtained from sample #14 belonged to a single cluster.

## DISCUSSION

Cases of *P. ovale* malaria have been reported in most countries in the Mekong sub-region, except in Cambodia.<sup>6-18</sup> Our study is therefore the first description of *P. ovale* in this country. The prevalence of *P. ovale* was nearly 4% in the 102 DNA samples examined here. Recent species-typing of 235 additional samples, collected during the same survey from the

inhabitants of two other villages in Rattankiri, showed a prevalence of 7–7.5%. These prevalences are higher than those reported from other countries of southeast Asia.<sup>10,13,14,17,18</sup> Our observations do not necessarily reflect the global situation of *P. ovale* in this country and call for large-scale surveys. Nevertheless, they indicate that *P. ovale* transmission occurs at a significant rate in northeastern Cambodia.

Compared with microscopy, the molecular approach offers a clear advantage for the characterization of minor species in mixed infections.<sup>11,12,14</sup> In our study, *P. ovale* was microscopically confirmed in only half of our samples, despite careful and lengthy examination of the slides. The low parasitemias of *P. ovale* and its presence in mixed infections make its microscopic identification very difficult, which is probably the main reason for its late discovery in Cambodia. Since this species was regularly reported in all surrounding countries, especially since the use of molecular diagnosis methods, we believe that *P. ovale* existed before in Cambodia but was simply overlooked. Further training of microscopists for the detection of this species on thin films is needed, and wherever possible molecular diagnosis methods should be used in combination with microscopy.



| geogr. origin | strains / isolates / clones                 | label in fig.3 | accession n°     | 853 | 855 | 957 | 1034 | 1038 | 1112 | 1115 | 1134           | 1135 | 1149 | haplotype | frequency         |                   |
|---------------|---|----------------|------------------|-----|-----|-----|------|------|------|------|----------------|------|------|-----------|-------------------|-------------------|
| Nigeria       | <i>Po</i> Nigerian/CDC strain, clone 9      | CDC1           | L48987           | -   | T   | A   | A    | C    | G    | G    | A              | A    | A    | CDC1      | 1/5               |                   |
| Nigeria       | <i>Po</i> Nigerian/CDC strain, clone 26     | CDC2           | L48986           | T   | C   | G   | T    | T    | A    | A    | A              | G    | G    | CDC2      | 1/5               |                   |
| Nigeria       | <i>Po</i> Nigerian/CDC strain               | CDC3           | AF145337         | -   | T   | G   | A    | C    | G    | G    | G              | A    | A    | CDC3      | 1/5               |                   |
| Cameroon      | <i>Po</i> isolates NGA, MAL/MAI             | CM             | AJ001527, X99790 | -   | C   | G   | A    | C    | G    | G    | G              | A    | A    | CM        | 2/5               |                   |
| Cambodia      | <i>Po</i> isolate 60, uncloned <sup>a</sup> | Cb 60          | NA               | T   | C   | G   | A    | T    | A    | A    | A              | G    | G    | Cb60      | 4/15 <sup>c</sup> |                   |
|               | <i>Po</i> isolate 14, clones A, B, D        | Cb 14A,B,D     |                  | -   | T   | A   | A    | C    | G    | G    | G              | A    | A    | A         | CDC1              | 3/15              |
|               | <i>Po</i> isolate 14, clone C               | Cb 14C         |                  | T   | C   | A   | A    | C    | G    | G    | C <sup>b</sup> | A    | A    | A         | Cb 14C            | 1/15 <sup>c</sup> |
|               | <i>Po</i> isolate 47, clones A, D, E        | Cb 47A,D,E     |                  | T   | C   | G   | A    | T    | A    | A    | A              | A    | G    | G         | Cb60              | (see isolate 60)  |
|               | <i>Po</i> isolate 47, clone B               | Cb 47B         |                  | -   | T   | G   | A    | T    | A    | A    | A              | A    | G    | G         | Cb 47B            | 1/15 <sup>c</sup> |
|               | <i>Po</i> isolate 47, clone C               | Cb 47C         |                  | -   | C   | A   | A    | C    | G    | G    | C <sup>b</sup> | A    | A    | A         | Cb 47C            | 1/15 <sup>c</sup> |
|               | <i>Po</i> isolate 94, clones A, E           | Cb 94A,E       |                  | T   | C   | G   | T    | T    | A    | A    | A              | A    | G    | G         | CDC2              | 2/15              |
|               | <i>Po</i> isolate 94, clone B               | Cb 94B         |                  | T   | C   | G   | A    | C    | G    | G    | C <sup>b</sup> | A    | A    | A         | Cb 94B            | 1/15 <sup>c</sup> |
|               | <i>Po</i> isolate 94, clone C               | Cb 94C         |                  | -   | T   | A   | T    | T    | A    | A    | A              | A    | G    | A         | Cb 94C            | 1/15 <sup>c</sup> |
|               | <i>Po</i> isolate 94, clone D               | Cb 94D         |                  | -   | T   | A   | A    | C    | G    | G    | C <sup>b</sup> | A    | A    | A         | Cb 94D            | 1/15 <sup>c</sup> |
| El Salvador   | <i>Pv</i> SAL 1 strain, A-type              | NA             | UO7367           | -   | -   | G   | A    | C    | G    | G    | G              | A    | A    | NA        | NA                |                   |
|               | <i>Pv</i> SAL 1 strain, S-type              | NA             | UO7368           | -   | -   | G   | A    | C    | G    | G    | G              | T    | A    | NA        | NA                |                   |
|               | <i>Pv</i> SAL 1 strain, O-type              | NA             | U93095           | -   | -   | G   | A    | C    | A    | G    | A              | G    | G    | NA        | NA                |                   |

FIGURE 2. Alignment of *Plasmodium ovale* (*Po*) and *P. vivax* (*Pv*) small subunit (SSU) ribosomal RNA (rRNA) sequences. The *P. ovale*-specific SSU rRNA polymerase chain reaction (PCR) products were obtained from four Cambodian isolates, cloned, and sequenced (<sup>a</sup> isolate 60: direct sequencing of the nested PCR product). The resulting sequences were aligned with previously published *P. ovale* and *P. vivax* SSU rRNA sequences (GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan accession numbers are listed). Shown are the nucleotides at the main polymorphic positions of the *P. ovale* SSU rRNA gene between nucleotides 837 and 1151 (positions according to the CDC2 sequence, accession number L48986), showing one new point mutation at position 1134 (<sup>b</sup>) and seven new haplotypes (<sup>c</sup>). The frequency of each haplotype among published and newly determined sequences is indicated. NA = not applicable. geogr. = geographic.

The sequencing of the *P. ovale* SSU rRNA PCR products provided undisputable confirmation of the species diagnosis in all four cases. It also showed unexpected sequence diversity, with the identification of nine different haplotypes, seven of which were novel. Cloning and sequencing showed up to four distinct *P. ovale* SSU rRNA sequences per sample. This is the first time that such high sequence diversity has been observed within individual *P. ovale* isolates, and it is particularly remarkable since the isolates all originated from a single village. Sequence variations in the SSU rRNA gene of *P. ovale* can have important implications on its molecular diagnosis with species-specific primers or probes. Indeed, failures of *P. ovale* and *P. malariae* detection because of minor nucleotide changes have been previously reported.<sup>15,16</sup> For probe-hybridization methods, the risk of misdiagnosis could be reduced by using multiple species-specific probes and a separate assay with a genus-specific probe. Larger studies of the entire SSU rRNA gene in wild *P. ovale* isolates of different geographic origins would be useful to get a better knowledge of the actual molecular diversity of this species.

However, our results also raise the question of the origin of such a high sequence diversity in individual *P. ovale* isolates. It is unclear whether it reflects the presence of multiple clonal types of *P. ovale* in each isolate, or the existence of distinct gene copies co-amplified from a single parasite clone, or a combination of both.

Previous reports from southern Vietnam, Myanmar, Laos, and Thailand described a single variant form of the *P. ovale* SSU rRNA gene, and mixed infections with the normal and the variant forms were observed.<sup>13,15,18</sup> Based on considerable sequence variations in the V7 and V8 regions of the SSU rRNA gene in different *P. ovale* isolates, the existence of *P. ovale* subspecies has even been evoked,<sup>27</sup> and more recent data seem to support the existence of at least two types of *P. ovale* variants or subpopulations.<sup>20,21,28</sup> According to these data, the sequences found in our study would rather belong to the "classic-type" variant, but with considerable variations

within this subgroup and also within individual isolates. However, variations in the *Plasmodium* SSU rRNA sequence are not restricted to species, subspecies, or variants. It has been shown that four to eight genomic rDNA units exist in *Plasmodium spp.* that consist of two to three distinct developmentally expressed gene sets with specific sequence signatures.<sup>29</sup> In particular, *P. falciparum* has A- and S- type SSU rRNA, being specifically transcribed during the asexual and sexual/sporozyte stages, respectively.<sup>30</sup> *Plasmodium vivax* has an additional O-type transcribed in the ookinete/oocyst stage.<sup>31</sup> For *P. malariae* and *P. ovale*, only a single SSU rRNA gene type has been described. The copy number of the SSU rRNA gene in *P. ovale* is unknown. Based on the copy number in other *Plasmodium* species, it could be approximately 4–8 per genome.<sup>29</sup> The primers used for our molecular analysis are specific for the SSU rRNA S-type in *P. falciparum*, for the SSU rRNA A-type in *P. vivax*,<sup>23</sup> and for an unknown type in *P. malariae* and *P. ovale*. Some of the diversity observed in the Cambodian *P. ovale* SSU rRNA sequences might result from amplification of more than one SSU rRNA gene type per genome. This hypothesis is supported by the phylogenetic analysis of the *P. ovale* SSU rRNA sequences shown in Figure 3. According to this analysis, the sequences derived from a single sample of the *P. ovale* reference strain Nigerian/CDC,<sup>32</sup> as well as those cloned from the Cambodian *P. ovale* isolates, divide into two major genetic clusters. Furthermore, the alignment with the A-, S- and O-type SSU rRNA genes of *P. vivax*, which is phylogenetically the closest *Plasmodium* to *P. ovale*,<sup>32</sup> showed similar patterns in two stage-specific *P. vivax* sequences and in the two *P. ovale* sequence types. We therefore tend to conclude that more than one sequence type per genome could have been amplified from the Cambodian *P. ovale* isolates, but the extent of sequence variation between these gene copies would not necessarily be as big as between the A-, S-, or O- gene types of *P. falciparum* and *P. vivax*. The question of how many distinct rRNA gene units were amplified remains open. We observed up to four distinct haplo-

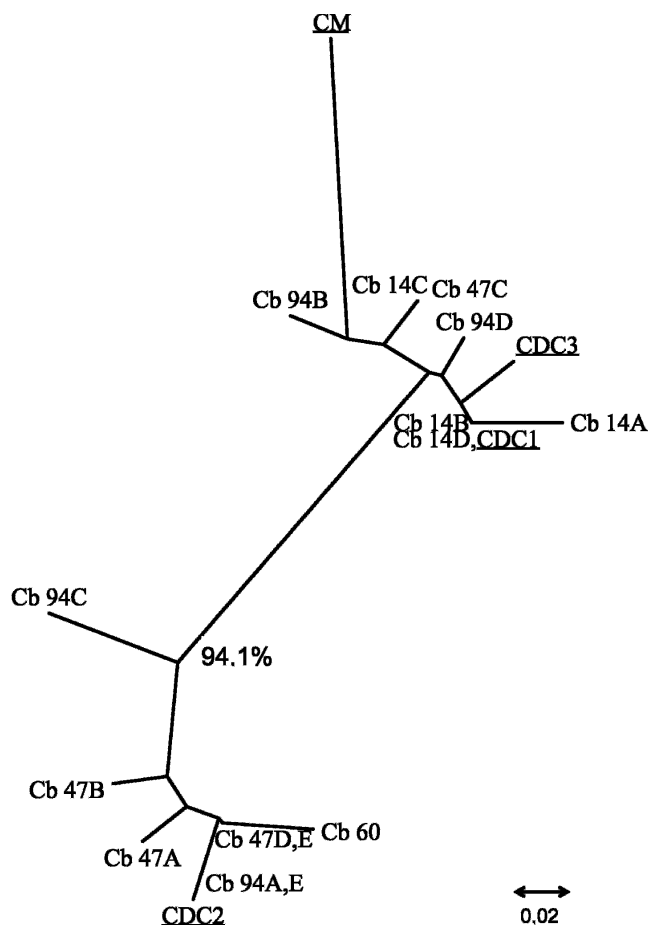


FIGURE 3. Clustering of *Plasmodium ovale* small subunit (SSU) ribosomal RNA (rRNA) sequences. An unrooted phylogenetic tree was derived from 4 published *P. ovale* SSU rRNA sequences (underlined) corresponding to the accession numbers AJ001527 (CM), L48987 (CDC1), L48986 (CDC2), and AF145337 (CDC3), and from 15 Cambodian sequences corresponding to cloned and uncloned *P. ovale*-specific SSU rRNA polymerase chain reaction products of the isolates #14 (Cb 14A to D), #47 (Cb 47A to E), #60 (Cb 60), and #94 (Cb 94A to E) by using the Phylip DNAdist program and the neighbor-joining method. Two major clusters were identified with a bootstrap reliability of 94.1% (1,000 replications). The bootstrap values of all other branches were below 70%, considered as the limit of statistical reliability. The scale bar indicates an evolutionary distance of 0.02 nucleotide substitutions per position.

types per sample, which may all represent distinct SSU rRNA units within a single genome. It has been reported that artificial genetic diversity can be generated by recombination between templates of related sequences during PCR amplification.<sup>33</sup> Indeed, the possibility that some of the *P. ovale* haplotypes were artificially produced by PCR-mediated recombination of at least two different sequence types cannot be ruled out. However, it seems not to be a major issue in our study, since the sequences of the *P. falciparum*, *P. vivax*, and *P. malariae* SSU rRNA perfectly matched the published reference sequences. In addition, only one of the nine observed SSU rRNA *P. ovale* haplotypes might actually have resulted from recombination of two other sequences in the same sample (Cb94B derived from Cb94A/E and Cb94D; Figure 2). Another possibility to be considered is that the different SSU

rRNA sequences could have originated from different *P. ovale* parasite clones. Given the low prevalence of *P. ovale* in the region, the probability of one patient being simultaneously infected with two or more genetically distinct *P. ovale* populations should be low, but it cannot be excluded that several *P. ovale* variants or subspecies are frequently transmitted together. The high sequence diversity observed in the examined isolates could even have resulted from a combination of polyclonality and intra-genomic variations. In summary, we conclude that substantial heterogeneity of *P. ovale* SSU rRNA sequences was observed between and within *P. ovale* isolates from northeastern Cambodia, and we suggest that SSU rRNA sequence diversity, usually interpreted as strain-to-strain variations, may well be due to variations among different SSU rRNA gene copies within a single genome. Further work is needed to examine this possibility, in particular sequence analysis of the *P. ovale* rRNA expressed at different developmental stages and analysis of the full *P. ovale* rDNA gene set.

Our results confirm that *P. ovale* is more prevalent in southeast Asia than previously suspected. This species probably remained undetected because of diagnosis difficulties. Indeed, PCR-based methods for the diagnosis of malaria have recently been introduced in Cambodia and proved useful for the detection of minor species and mixed infections. This is an important issue since interactions between species in the same patient may affect both the course and the severity of the infection, as well as the response to treatment.<sup>34-37</sup> Our observations call for larger community-based surveys, using microscopy and molecular diagnosis methods, to obtain more accurate information about the epidemiologic situation of malaria in Cambodia. Indeed, a precise estimate of the prevalences of the four malaria species and the frequency of mixed infections is required to optimize the medical treatments and malaria control measures according to the local characteristics of the disease.

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