tify the closest relative of each human *Plasmodium* species, investigations on molecular phylogeny are essential.

Plasmodium phylogeny is still controversial. Optimal trees reconstructed using different genes are not always consistent with each other. Previous attempts for phylogenetic reconstruction of the genus *Plasmodium* have used several molecular markers such as cytosolic small subunit rRNA (cyto-SSU rRNA or 18S rRNA) (e.g. Qari et al., 1996; Escalante et al., 1997; Leclerc et al., 2004; Rooney, 2004), mitochondrial cytochrome B (CytB) (Escalante et al., 1998; Perkins and Schall, 2002), apicoplast caseinolytic protease (ClpC) (Rathore et al., 2001), circumsporozoite protein (CSP) (McCutchan et al., 1996; Escalante et al., 1995; Vargas-Serrato et al., 2003), and merozoite surface antigen 9 (MSP9) (Vargas-Serrato et al., 2003). There are two phylogenetic analyses that simultaneously consider more than one gene (Escalante et al., 2005; Perkins et al., 2007). Until recently, based on these phylogenetic analyses, the monophyly of simian (non-hominoid) *Plasmodium* species and *P. vivax* (referred to as 'simian *Plasmodium* + *P. vivax* clade' in this report) and the monophyly of rodent *Plasmodium* species ('rodent *Plasmodium* clade') have been demonstrated.

Among the molecular markers described above, cyto-SSU rRNA has been examined most frequently and many trees have been proposed (e.g. Qari et al., 1996; Escalante et al., 1997; Leclerc et al., 2004; Rooney, 2004). Since cyto-SSU rRNA is the most common marker generally applied in the field of eukaryotic phylogeny, and with a wealth of sequence data reported from various *Plasmodium* species, phylogenetic inference of the *Plasmodium* tree based on the cyto-SSU rRNA sequences is always important and indispensable, especially when it entails the analysis and positioning of a new species. There are, however, inherent problems in using cyto-SSU rRNA genes in reconstructing *Plasmodium* phylogeny. Unlike most of the eukaryotic organisms analyzed so far, the *Plasmodium* species have divergent, paralogous copies of cyto-SSU rRNA gene. For example, in *P. falciparum*, sequence difference among the multi-copy genes amounts to as high as 11% (McCutchan et al., 1988). Different genes are expressed at various stages of the parasite life cycle. The presence of asexual- (A-) and sporozoite- (S-) types has been reported in *P. falciparum*, *P. berghei*, *P. vivax*, and several other species (Rogers et al., 1998; van Spaendonk et al., 2001). An additional oocyst- (O-) type has also been reported in *P. vivax* (Li et al., 1997). These gene duplication events could potentially complicate inference of *Plasmodium* phylogeny, because an inferred tree always reflects the history of gene duplication and speciation events. However, this issue has not been confronted in previous attempts to infer a species tree, with most reports largely focusing only on the A-type-like genes (Qari et al., 1996; Leclerc et al., 2004).

Recently, the underlying mechanism maintaining different copies of cyto-SSU rRNAs in apicomplexan parasites, the genus *Plasmodium* and the genus *Cryptosporidium*, has been investigated (Rooney, 2004). In the simian *Plasmodium* + *P. vivax* clade of the *Plasmodium* tree, different SSU rRNA genes were found to form three clades in a between-species clustering manner, which was explained by a birth-and-death model under strong purifying selection. In this model, new genes are created by gene duplication and some duplicate genes stay in the genome for a long time, while others are inactivated or deleted from the genome. The model is in contrast to the concerted evolution model, in which all member genes of a family evolve as a unit in concert (for review see Nei and Rooney, 2005). Since eukaryotic rRNA genes were generally considered to evolve in a concerted manner, the finding that cyto-SSU rRNAs of the genus *Plasmodium* evolve in the birth-and-death manner was a novel insight. However, even though Rooney (2004) used all *Plasmodium* cyto-SSU rRNA sequences available at that time, the *Plasmodium* species originating from simian taxa were limited in number. In this case, we wondered whether additional patterns not detected in Rooney (2004) might be revealed through further analysis of the cyto-SSU rRNA phylogeny when genes from additional species are considered, especially in the simian *Plasmodium* + *P. vivax* clade.

In this work, we cloned and sequenced the cyto-SSU rRNA genes from eight simian *Plasmodium* species (*P. gonderi*, *P. fragile*, *P. coatneyi*, *P. inui*, *P. hylobati*, *P. fieldi*, *P. simiovale*, and *P. cynomolgi*). Natural host and geographic distribution of these species are summarized in Table 1, together with those of *P. knowlesi* and *P. vivax*. *P. gonderi* is found in African monkeys while other species are found in Asian monkeys mainly in *Macaca* spp. *P. hylobati* is considered to have switched its host from macaques to gibbons (Mu et al., 2005). The human parasite, *P. vivax*, has been shown to be closely related to these simian parasites, but its closest relative has not been clearly established (Escalante et al., 2005). Close relationships between *P. inui* and *P. hylobati*, between *P. coatneyi* and *P. knowlesi*, and between *P. simiovale* and *P. fieldi* and the earliest branching status of *P. gonderi* in the simian *Plasmodium* tree have been suggested by several different phylogenetic markers (Perkins and Schall, 2002; Vargas-Serrato et al., 2003; Leclerc et al., 2004; Escalante et al., 2005; Tanabe et al., 2007). However, the positions of other species and the branching order among the simian *Plasmodium* + *P. vivax* clade, except for *P. gonderi*, remains to be determined.

Here, we report the sequences of A- and S-type-like cyto-SSU rRNA genes of the above eight simian *Plasmodium* species. Detailed alignment analysis together with exploration of the secondary structure model of the *P. vivax* A-type cyto-SSU rRNA demonstrated an approximately 50-residue insertion in the V7 variable region near the stem 43 is shared exclusively by the S-type-like sequences of the Asian simian *Plasmodium* species and the S- and O-type sequences of *P. vivax*. Phylogenetic analyses including all sequences from the human, simian, rodent, and avian *Plasmodium* species demonstrated that

Table 1
Simian *Plasmodium* species and *P. vivax* with their host and accession numbers for the cytosolic SSU rRNA sequences determined in this study

Species	Host	Geographic distribution	Accession No.
P. gonderi	*Cercocebus atys* *Cercopithecus* spp.	Central Africa	AB287269 AB287270 AB287271
P. fragile	*Macaca radiat,* *M. sinisca*	Southern India Sri Lanka	AB287272 AB287273
P. coatneyi	*M. fascicularis*	Malaysia Philippines	AB265789 AB265790 AB265791
P. knowlesi	*M. fascicularis* *M. nemestrina* *Presbytis melalophos* *Homo sapiens*	Southeast Asia	
P. inui	*Cynopithecus niger* *M. cyclopis* *M. fascicularis* *M. nemestrina* *M. mulatta* *M. radiata* *P. cristatus* *P. obscurus*	South and East Asia	AB287275 AB287276 AB287277
P. hylobati	*Hylobati moloch*	Indonesia Malaysia (Borneo)	AB287278 AB287279 AB287280
P. fieldi	*M. nemestrina* *M. fascicularis*	Malaysia	AB287281 AB287282 AB287283 AB287284
P. simiovale	*M. sinica*	Sri Lanka	AB287285 AB287286 AB287287
P. cynomolgi	*M. fascicularis* *M. nemestrina* *M. radiata* *M. cyclopis* *M. sinica* *M. mulatta* *P. entrellus* *P. critatus*	Southeast Asia	AB287288 AB287289 AB287290
P. vivax	Human	Tropics	Worldwide

Note. Natural hosts and geographic distributions are found in Coatney et al. (1971).

gene duplication events giving rise to A- and S-type-like sequences took place independently at least three times in the evolution of *Plasmodium*.

2. Material and methods

2.1. Sample collection

Genomic DNAs of *P. gonderi*, *P. fragile* (Hackeri strain), *P. inui* (Celebes strain), *P. hylobati*, and *P. simiovale* was isolated from whole blood using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). Infected blood samples of *P. fieldi* (N-3 strain) and *P. cynomolgi* [M (Mulligan) strain] were obtained from ATCC (no 36163 and no 30155, respectively), and genomic DNAs were prepared using a QIAamp DNA Blood mini Kit (QIAGEN, Hilden, Germany) according to the method described by Sakihama et al. (2001). *P. coatneyi* (CDC strain) was provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA), and animal infection was performed according to the procedures described by Matsumoto et al. (2000) and Kawai et al. (2006). Parasite infected blood sample was passed through a leukocyte reduction filter (Immugard® III-RC; TERUMO, Tokyo, Japan) and washed three times with RPMI 1640 medium. Genomic DNA of *P. coatneyi* was extracted with the use of DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

2.2. Amplification and sequencing

SSU rRNA genes were amplified by PCR using Pfu DNA Polymerase (Promega, Madison, WI, USA) and two sets of primers of which sequences are common in A- and S-type genes: the first primers were PlaSSU5 and PlaSSU3r, the second (nested) primers were SSUF1 and SSUR1 (Supplementary Table 1). A reaction mixture of 16.5 μl contained 2.5 mM of each dNTP, 1 μM of each primer, 0.5 U of Pfu DNA Polymerase, 0.5 μg DNA template, and 1× reaction buffer (Promega, Madison, WI, USA). After initial denaturation at 95 °C for 2 min, PCR was performed for 30 cycles of 15 s at 95 °C, 30 s at 60 °C, 5 min at 68 °C with a final extension of 10 min at 68 °C. The nested PCR cycling condition was 95 °C for 2 min, followed by 30 cycles of 15 s at 95 °C, 30 s at 59.3 °C, 5 min at 68 °C with a final extension of 10 min at 68 °C.

The amplified products were cloned into pCR-Blunt II-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Plasmid DNAs containing the SSU rRNA genes were prepared using QIAGEN Plasmid Mini kit (QIAGEN, Hilden, Germany). Ten clones for each species with approximately 2 kb inserted fragments were sequenced on an ABI 3100 Genetic Analyzer using ABI BigDye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems, Foster City, CA, USA).

Since we could not completely exclude the possibility that cyto-SSU rRNA sequences obtained here might be artifacts due to in vitro recombination events during cloning (Tanabe et al., 2002), sequences were confirmed using the direct sequencing approach. A- and S-type specific primers were designed in the vicinity of the unique insertion/deletion region based on the SSU rRNA sequences of each *Plasmodium* species determined in this study (Supplementary Table 1). The PCR condition was the same as the one described above for amplifying approximately 2 kb fragments. PCR products were purified on MicroSpin Columns prepared with Sephacryl S-300 HR resin (Amer-

sham BioSciences, Sweden) and sequenced on an ABI 3100 Genetic Analyzer using ABI BigDye Terminator Cycle Sequencing kit v1.1. The simian *Plasmodium* SSUrRNA sequences reported in this study were deposited in GenBank, with the Accession Nos. AB265789–AB265791 and AB287269–AB287290.

2.3. Sequence alignment and phylogenetic analyses

The cyto-SSU rRNA sequences of the simian *Plasmodium* species were aligned with those of other *Plasmodium* species obtained from GenBank and TIGR databases. The species name and accession numbers are given in the resultant trees (see Figs. 1 and 2). The alignment of the genus *Plasmodium* was based on the secondary structure of the molecule available in the European ribosomal RNA database (Wuyts et al., 2004), and was manually optimized with other database sequences and originally reported sequences (shown in supplementary Fig. 1). The secondary structure model of *P. vivax* (U03079, A-type) at the website (http://www.psb.ugent.be/rRNA/secmodel/Pviv_SSU.html) was used for searching the location of insertion/deletion region characteristic to A-, S-, and O-type genes.

Two data sets with different taxon samplings were generated from the alignment. One included 58 cyto-SSU rRNA sequences of the *Plasmodium* species from humans, simians, rodents, and birds, while the other included 37 sequences, which only consisted those from simian *Plasmodium* species and from *P. vivax*.

Phylogenetic analyses were performed with neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods using PAUP 4.0β10 (Swofford, 2002) program. Prior to the NJ and ML analyses, 56 different substitution models were compared by using MODELTEST3.7 (Posada and Crandall, 1998) program. The GTR (Rodriguez et al., 1990; Yang, 1996) + Γ + *I* model and HKY85 (Hasegawa et al., 1985) + Γ + *I* model were selected as the best models for the two data sets with 58 and 37 sequences, respectively. Parameters estimated by the MODELTEST 3.7 program were assumed for inferring phylogenetic trees. The parameter estimates were confirmed to be comparable with those estimated during the inference of the ML tree. The ML and MP trees were reconstructed under heuristic searches using tree bisection reconnection (TBR) branch swapping in which the starting tree was obtained via stepwise addition. Nodal support was estimated by using non-parametric bootstrap (BP) (Felsenstein, 1985) with 100 replications for ML analyses, and 1000 replications for MP and NJ analyses. In the ML analyses, to evaluate the support of a given tree among alternative trees, *p*-values generated from an approximately unbiased AU test (Simodaira, 2002) were used for comparing log-likelihoods among alternative trees. The programs, PAML 3.15 (Yang, 1997) and CONSEL (Shimodaira and Hasegawa, 2001), were used for the comparison analysis.

3. Results

3.1. cyto-SSU rRNA sequences of the simian Plasmodium species

Nucleotide sequences of the cyto-SSU rRNA genes from eight simian *Plasmodium* species were obtained by cloning and direct sequencing of corresponding PCR products. Based on sequence alignment analysis, we found a single S-type-like sequence and two to three A-type-like sequences for each Asian simian *Plasmodium* species (Supplementary Fig. 1). The S-type-like sequences of Asian simian *Plasmodium* species and *P. vivax* have a unique insertion of approximately 50 residues in the V7 variable region near the stem 43 (e.g. Van de Peer et al., 2000), while no such insertion was found in their A-type-like sequences. An insertion at this region was observed also for the O-type sequence of *P. vivax*. On the other hand, no such distinctive insertion was observed in the cyto-SSU rRNA sequences of other *Plasmodium* species including the African simian *Plasmodium* species *P. gonderi* (Supplementary Fig. 1).

3.2. cyto-SSU rRNA phylogeny of the simian Plasmodium + P. vivax clade

The data set of 37 cyto-SSU rRNA sequences including simian *Plasmodium* species and *P. vivax* consisted of 1618 sites, with 332 variable and 165 parsimony informative sites. Redundant identical sequences that had been generated in the process of selecting the 1618 sites from an original alignment were removed from the data set, and then 35 different sequences were analyzed. The ML tree revealed monophyly of all S-type-like sequences and monophyly of all A-type-like sequences, placing the *P. vivax* O-type sequence within the A-type-like clade (Fig. 1). In the S-type-like clade, close relationships were recovered for *P. inui–P. hylobati*, *P. fieldi–P. simiovale*, and for Asian simian *Plasmodium* species. In contrast, for the A-type-like clade, no clear resolution was obtained for most of the subtree. Also, in the A-type-like clade, two to three different sequences in a given species were closely related with each other to form a clade that excluded the sequences from other species. The position of the O-type sequence of *P. vivax* in the clade of A-type-like sequences is yet unclear, as will be described below.

To address the influence of the extremely long branch O-type sequence on the obtained phylogenetic tree in Fig. 1, we reconstructed a tree using the data set from 34 simian *Plasmodium* sequences excluding the *P. vivax* O-type sequence. Omission of the O-type sequence did not produce an optimal ML tree that was significantly different from the one including the O-type sequence (data not shown).

3.3. cyto-SSU rRNA phylogeny of the genus Plasmodium

The data set of 58 SSU rRNA sequences from parasites from simians, humans, birds, and rodents consisted of 1419

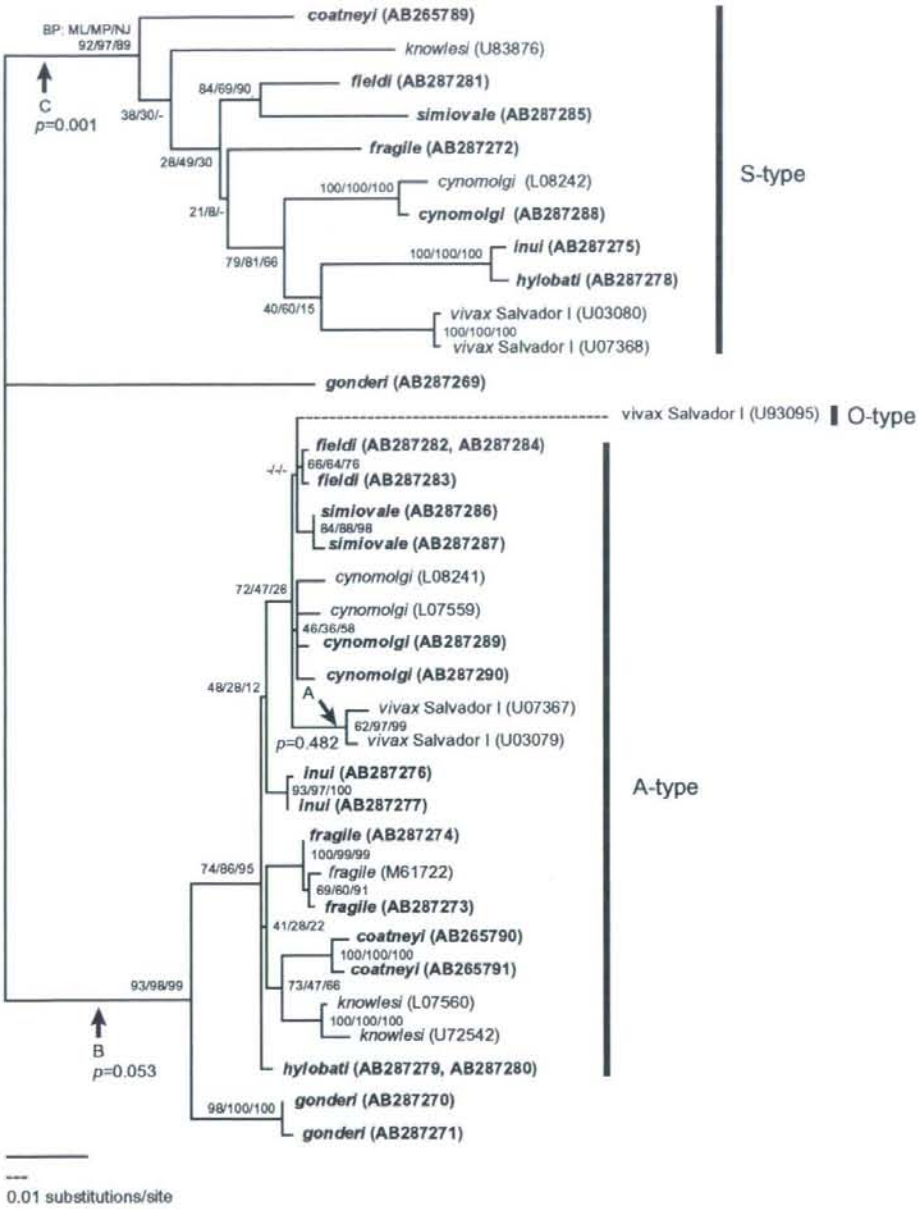

Fig. 1. Optimal ML tree of simian *Plasmodium* species and *P. vivax*. The phylogeny was inferred based on the HKY + I + Γ model from the selected 1618 sites. Bootstrap values for the ML tree are shown with those calculated by MP and NJ methods (ML/MP/NJ). '-' denotes BP < 5%. Estimates for proportion of invariant sites (I) and Γ-shape parameter (α) are $I = 0.6038$ and $\alpha = 0.6788$. The branch lengths are drawn proportional to the amount of changes (scale shown). Arrows, A–C, indicate alternative positions of the O-type sequence compared by the AU test (see text, the alternative positions within the S-type-like clade are not shown). p-values of the AU test are also shown. In order to carry out AU test, the trifurcated topology of the subtree including the O-type sequence, (vivax-O, (fieldi, fieldi), (simiovale, simiovale)), in the optimal tree was replaced by a bifurcated one, (vivax-O, ((fieldi, fieldi), (simiovale, simiovale))). Then the O-type sequence was constrained to other positions. All *Plasmodium* species are designated with their corresponding species name and accession number. Data obtained in this study are denoted in bold.

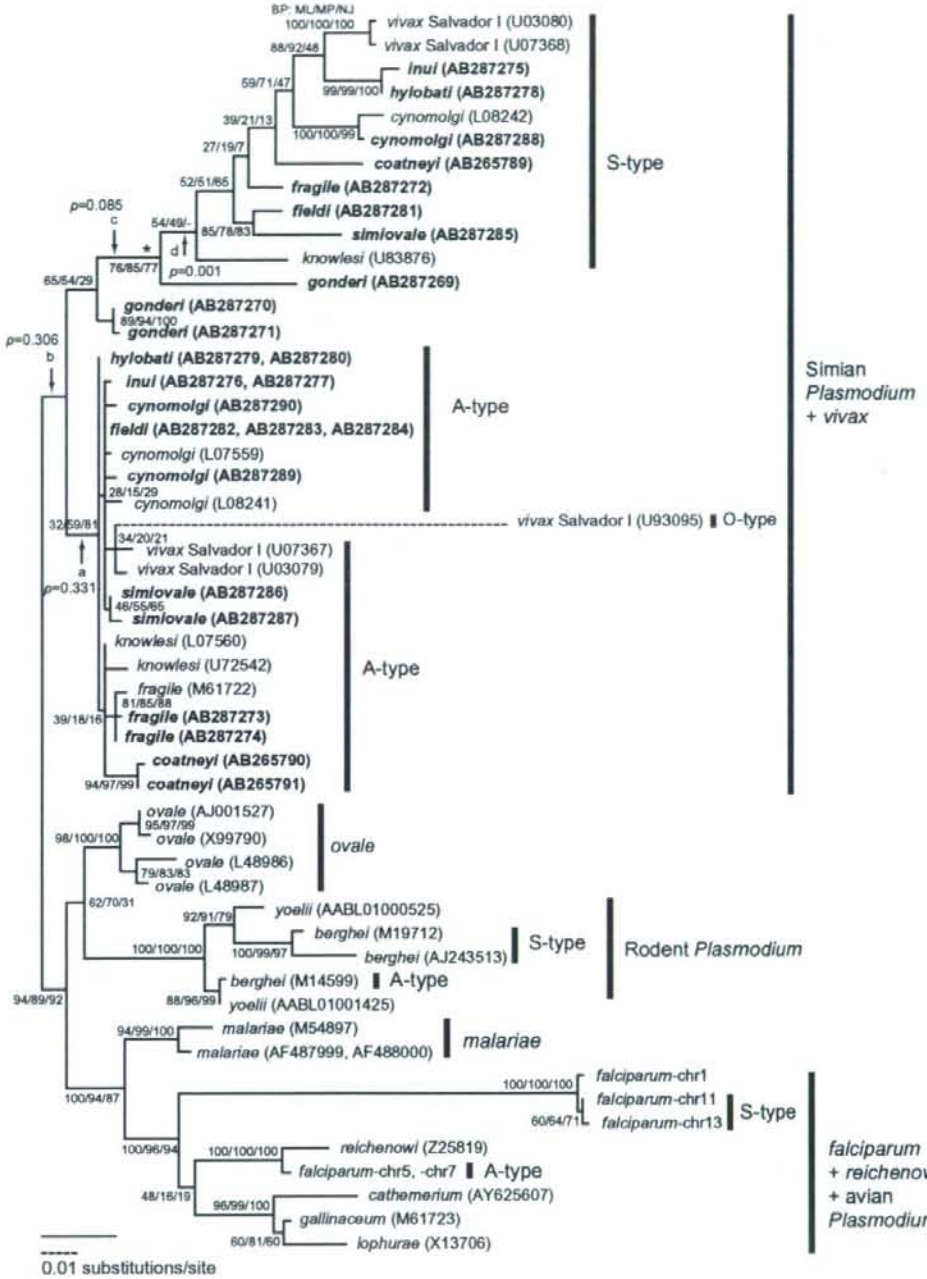

Fig. 2. Optimal ML tree of the genus *Plasmodium*. The phylogeny was inferred from the selected 1419 sites. Bootstrap values in the ML tree are shown with those calculated by MP and NJ methods (ML/MP/NJ). '-' denotes BP < 5%. Estimates for proportion of invariant sites (I) and Γ-shape parameter (α) are $I = 0.5662$ and $\alpha = 0.7493$. Arrows, a–d, indicate alternative positions of O-type sequence compared by the AU test (see text, the alternative positions within the S-type-like clade are not shown). p-values of the AU test are also shown. In order to carry out AU test, the trifurcated topology of the subtree including the O-type sequence (vivax-O, vivax-A, vivax-A), in the optimal tree was replaced by a bifurcated one (vivax-O, (vivax-A, vivax-A)). Then the O-type sequence was constrained to other positions. *An alternative root position of the simian *Plasmodium* + *P. vivax* clade. For details, see also the legend to Fig. 1.

sites, with 312 variable and 164 parsimony informative sites. Removing redundant identical sequences, 52 sequences were analyzed. The optimal ML tree (Fig. 2) explicitly recovered monophyletic relationships of the rodent *Plasmodium* clade (100% BP in the ML analysis), the *P. malariae* clade (94% BP), the *P. ovale* clade (98% BP), and the simian *Plasmodium* + *P. vivax* clade (94% BP). The tree clearly supported also a monophyletic relationship for the avian *Plasmodium* clade and the *P. falciparum*/*P. reichenowi* clade (100% BP), and a sister-group relationship of the *P. malariae* clade to the large clade of the avian *Plasmodium* + *P. falciparum*/*P. reichenowi* (100% BP) (Fig. 2).

The ML tree demonstrated that the root of the simian *Plasmodium* + *P. vivax* clade was located on the common ancestor of the sequences of the A-type-like clade, although BP support for this rooting was low (65%) (Fig. 2). We investigated a possibility for an alternative root position as shown by the asterisk in Fig. 2, which separates two types of the *P. gonderi* sequences. The *p*-values of the AU test for the alternative rooting was $p = 0.091$, revealing that the possibility cannot be rejected with this data set.

3.4. Position of the O-type sequence

We evaluated alternative possibilities for the position of O-type sequence using the AU test. On the backbone tree in Fig. 1, the branch leading to the O-type sequence was constrained to the alternative positions: (A) the branch for the common ancestor of the A-type sequences of *P. vivax*, (B) the branch for the common ancestor of the A-type-like sequences of the Asian simian *Plasmodium* species and the *P. gonderi* AB287270/AB287271 sequences, and (C) the branch for the common ancestor of the S-type-like sequences, and all branches within the S-type-like clade. As shown in the tree in Fig. 1, the trees that constrained the O-type sequence to the positions (A) and (B) were not statistically significantly different from the optimal tree, while all other trees were significant ($p < 0.05$, data not shown). Similarly, on the backbone tree in Fig. 2, the branch leading to the O-type sequence was constrained to the alternative positions: (a) the branch for the common ancestor of the A-type-like sequences, (b) the branch for the root of the simian *Plasmodium* + *P. vivax* clade, (c) the branch for the common ancestor of the *P. gonderi* AB287269 sequence and the S-type-like sequences, and (d) the branch for the common ancestor of the S-type-like sequences, and all branches within the S-type-like clade. The AU test indicated that the trees with the O-type sequence at the alternative positions (a) through (c) were not statistically significantly different from the optimal tree, while other trees were significantly different ($p < 0.05$, data not shown). These results suggest that the *P. vivax* O-type sequence is unlikely to have originated from an S-type-like sequence by gene duplication(s), although both of these gene types share the 50-residue insertion.

4. Discussion

In the present study, we sequenced multiple genes encoding cytosolic SSU rRNA of eight simian *Plasmodium* species, and performed phylogenetic analyses of these and previously published sequences. The ML phylogeny of the simian *Plasmodium* + *P. vivax* clade clearly revealed the *P. inui*–*P. hylobati* and *P. simiovale*–*P. fieldi* relationships (Fig. 1). These further strengthen the results obtained using a concatenated β-tubulin, cdc-2, and plastid *tuf*A data set (Escalante et al., 2005). The close affinity of *P. inui* with *P. hylobati* has been revealed also by the phylogenies of mitochondrial Cyt B (Perkins and Schall, 2002) and MSP1 (Tanabe et al., 2007). Although not clearly supported by BP analyses, the close relationship between *P. coatneyi* and *P. knowlesi* was reconstructed in the A-type-like clade in the tree in Fig. 1, comparable to the previous results obtained from phylogenies of CSP and MSP9 (Vargas-Serrato et al., 2003), MSP1 (Tanabe et al., 2007), and the concatenated β-tubulin, cdc-2, and plastid *tuf*A data set (Escalante et al., 2005).

In the unrooted tree of the simian *Plasmodium* + *P. vivax* clade (Fig. 1), separation between the A- and the S-type-like clades for Asian simian sequences was clearly supported (Fig. 1), placing the *P. vivax* O-type within the A-type-like clade. Although we could not confirm the types, A or S, for three sequences from the African simian *Plasmodium* species *P. gonderi*, if two closely related sequences, AB287270 and AB287271, were A-type, while a relatively divergent sequence, AB287269, was S-type, then a gene duplication event would likely have occurred in the common ancestor of simian *Plasmodium* species, generating the ancestors of the S- and the A-type-like sequences. Although the rooted tree (Fig. 2) did not locate the root between the *P. gonderi* AB287269 sequence and the *P. gonderi* AB287270/AB287271 sequences, this possibility could not be rejected with statistical significance, and thus cannot be ruled out. In the simian *Plasmodium* + *P. vivax* clade in Figs. 1 and 2, closely related A-type-like sequences were found in multicopies in all branches of simian *Plasmodium* species examined, suggesting that independent gene duplications occurred after the divergence of all simian *Plasmodium* species.

Since the possibility for the S-type relationship of the *P. vivax* O-type sequence was excluded by statistical test, shared insertions by the *P. vivax* O- and S-types (Supplementary Fig. 1) should have been acquired independently on the branch leading to the common ancestor of S-type-like sequences and the branch leading to the *P. vivax* O-type sequence. However, an alternative possibility exists that homologous recombination of the partial sequence flanking the insertion/deletion region may have occurred between the S- and A-type genes of *P. vivax* and thus resulted in the formation of the ancestor of O-type gene. The presence of O-type gene has been confirmed on the genome sequencing database (Accession No. AAKM01002769), where a sequence of more than 99% similarity to the O-type gene

(U93095) exists. Thus, it is highly unlikely that the *P. vivax* O-type gene is an artificial recombination product.

In the analysis of the tree in Fig. 2, in which the simian *Plasmodium* + *P. vivax* clade was rooted by other *Plasmodium* sequences, neither the root of the tree nor the phylogenetic relationships among sequences could clearly be established, probably because of the lack of phylogenetic information resided in the restricted number of sites used. If the branching position of the *P. vivax* O-type was at (a), (b), or (c) in Fig. 2, then other simian *Plasmodium* species might also possess O-type genes. However, no O-type-like sequence has been reported so far from other simian *Plasmodium* species. Further sequence analyses are necessary to settle whether or not O-type-like genes are present in simian *Plasmodium* species. Of the alternative trees on different branching positions of the *P. vivax* O-type, the tree locating it at the position (b) in Fig. 2 is congruent with Li et al. (1997) maximum parsimony tree. Analyzing only limited number of *Plasmodium* sequences, the BP support of the early branching status of the *P. vivax* O-type in their tree amounted to 95% and thus the support was significant. Since the estimated branch length leading to the *P. vivax* O-type was extremely long (Figs. 1 and 2), the tree of Li et al. (1997) is most likely affected by a long branch attraction artifact. Although our analysis could not exclude the tree with statistical significance, the tree is less likely than our best tree (Fig. 2).

Similar to the simian *Plasmodium* + *P. vivax* clade, a gene duplication event that produced S- and A-type-like genes predated the speciation event both in the avian + *P. falciparum*/*P. reichenowi* clade and in the rodent *Plasmodium* clade (Fig. 2). Thus, at least three gene duplications that gave rise to the S- and A-type-like cyto-SSU rRNA genes occurred independently during *Plasmodium* evolution, supporting Rooney's (2004) hypothesis that the evolution of cyto-SSU rRNA genes of the genus *Plasmodium* can be explained by the birth-and-death model (Rooney, 2004). In either of these three clades, branch lengths leading to the S-type-like sequences were significantly longer than those of the A-type-like sequences (likelihood ratio test, $p < 0.01$, data not shown), indicating that evolutionary rates of the S-type-like sequences are accelerated, compared to those of the A-types. Functional constraints on the cyto-SSU rRNA might be different between the two types, plausibly because the A-types are expressed in vertebrate hosts, while the S-types in mosquitoes. An extremely long branch of the *P. vivax* O-type sequence (Fig. 1) suggests that the O-type cyto-SSU rRNA is functionally divergent. Since scarce information is available on the types of genes on *P. ovale*, *P. malariae*, and avian *Plasmodium* species, further characterization of the genes in these species is likely to be important for understanding the overall evolutionary history of cyto-SSU rRNA genes in the genus *Plasmodium*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.01.031.

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プロジェクト７：その他

インフルエンザ疾患

MINISTRY OF HEALTH

NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY

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PROJECT REPORT

CIRCULATION OF INFLUENZA LIKE ILLNESS IN CAM GIANG DISTRICT, HAI DUONG PROVINCE, 2009

HANOI, FEBRUARY 2009

THE FIRST QUARTER REPORT

1. Project title:

- Circulation of Influenza like illness in Cam Giang district, Hai Duong province, 2009

2. Host agency:

- Ministry of Health, Viet Nam

3. Implementation agency:

- National Institute of Hygiene and Epidemiology, Viet Nam

4. Study site:

- Cam Giang district, Hai Duong province

5. Research duration:

- 1 year (2009)

6. Study budget:

- Total funding for the project is 2.000.000 JPY equivalent to 376.000.000 VND
- Funding status: NIHE have received all of 2.000.000 JPY
- National Institute of Infectious Diseases, Japan as project sponsor

7. Implementation activities

7.1. Completed work

Administration approval: The project has been translated to Vietnamese and adapted with the legal and scientific forms to submit IRB, Scientific Committee at National Institute of Hygiene and Epidemiology, Viet Nam for reviewing and approval. And then the project document has been also submitted to Ministry of Health for reviewing and approval

An official letter from the National Institute of Hygiene and Epidemiology signed by Director has been sent to Provincial Preventive Medicine Center of Hai Duong province to request their collaboration, authority support and approval from Provincial People Committee.

List of 28 health staff from 19 communes in Cam Giang district, 3 health officers from Preventive Medicine Center of Cam Giang district and 3 health officers of Provincial Preventive Medicine Center of Hai Duong province are formulated in which the name, address, individual contact and position of all those collaborators are requesting to complete and send to the National Institute of Hygiene and Epidemiology and Provincial Preventive Medicine Center of Hai Duong province for supervision and management.

A plan for training course is proposed to conduct after getting an official approval from Ministrial level. The training course will be organized in the Preventive Medicine Center of Cam Giang district to train health staff for 2 days on the objective of the survey, survey method, mechanism of management and reporting, logistic arrangement.

A list of equipment, materials used for the survey is also generated and will be distributed to 19 Commune Health Stations, Preventive Medicine Center of Cam Giang district and Provincial Preventive Medicine Center of Hai Duong province.

Implementation plan is also built with the timing schedule to define individual works during 12 months.

Unimplemented works and plan

- To examine the prevalence of influenza like illness in Cam Giang district, Hai Duong province.
    - Carry out the training plan
    - Organize and implement the field work:
        - Questionnaire field testing
        - Routine identification of defined cases and conduct the interview and data and sample collection.
        - Supervision and management activities
    - Manage, store and enter data.
    - Direct the field activities
    - Analyse sample and data
    - Periodically complete report

  To evaluate the sensitivity and specificity of existing influenza and influenza like illness surveillance system at district level.
    - Collect influenza like illness cases in Cam Giang district, Hai Duong province from 2004 – 2008 and 2009
    - Use statistical software to evaluate the sensitivity and specificity of existing influenza like illness surveillance system at district level.
    - Complete summary report

8. Financial report

Fund source	Currency unit	Average rate of exchange in report year	Yearly process of investment	Process of investment of report year	Cummulative process of investment from the beginning of project	Proportion (%) of fact process of investment to yearly plan
(1)	(2)	(3)	(4)	(5)	(6)	(7)=(5)*100/(4)
Sponsor	JPY	1 JPY=188 VND	2.000.000 JPY =376.000.000 VND	2.000.000 JPY =376.000.000 VND	2.000.000 JPY =376.000.000 VND	0.0%

NIHE has been received all the funding from the National Institute of Infectious Diseases, Japan for one-year porject. All funding has not yet been reimbursed until having an approval from Ministry of Health. Therefore, all activities will be delayed until its approval pass.

Hanoi, dated February 28th, 2009

Assoc. Prof. Nguyen Tran Hien, MD., MPH., PhD
Project Director

Dr. Tham Chi Dung, MD., MS.
Reporter

平成２０年度業績

＊研究成果の刊行に関する一覧表

＊学会発表一覧表

研究成果の刊行に関する一覧表 （平成２０年度）

執筆者氏名	刊行書籍又は雑誌名 （雑誌のときは雑誌名、 巻号数、論文名）	刊行書店名	巻名	ページ	刊行年
プロジェクト１：細菌					
Kam KM, Luey CK, Parsons MB, Cooper KL, Nair GB, Alam M, Islam MA, Cheung DT, Chu YW, Ramamurthy T, Pazhzni GP, Bhattacharya SK, Watanabe H, Terajima J, Arakawa E, Ratchtrachenchai OA, Huttayananont S, Ribot EM, Gerner-Smidt P, Swaminathan B	Evaluation and Validation of a PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocol for Subtyping Vibrio parahaemolyticus: an International Multicenter Collaborative Study.	J Clin Microbiol	46 (8)	2766-2773	2008
プロジェクト２：ウイルス（デング熱）					
青山幾子、弓指孝博、加瀬哲男、高橋和郎、宇野健司、後藤哲志、片山智香子、中村匡宏、塩見正司、仁科展子、齊藤武志、森　登志子、穴瀬文也、吉田英樹、高崎智彦、林　昌宏、倉根一郎	チクングニヤ熱と確定診断されたインドからの輸入感染症症例	Infectious Agent Surveillance Reports	29	345-346	2008

Takasaki, T., Kotaki, A., Nishimura, K., Sato, Y., Tokuda, A., Lim, C.K., Ito, M., Tajima, S., Nerome, R., and Kurane, I.	Dengue virus type I isolated from an imported dengue patient in Japan: first isolation of dengue virus from Nepal.	Journal of Travel Medicine	15	46-49	2008
Tajima, S., Takasaki, T., and Kurane, I.	Characterization of Asn130-to-Ala mutant of dengue type 1 virus NS1 protein.	Virus Genes	36	323-329	2008
プロジェクト４：ウイルス（風疹と CRS）					
Sakata, M., Komase, K., and Nakayama, T.	Histidine at position 1042 of the p150 region of a KRT live attenuated rubella vaccine strain is responsible for the temperature sensitivity.	Vaccine	7:27(2)	234-42	2009
駒瀬勝啓	風疹ワクチンの効果と再感染	臨床とウイルス	36(1)	32-38	2008
駒瀬裕子、駒瀬勝啓	インフルエンザ	Medical Practice	25(5)	787-793	2008
Chan-It W, Khamrin P, Saekhow P, Pantip C, Thongprachum A, Peerakome S, Ushijima H, Maneekarn N.	Multiple combinations of P[13]-like genotype with G3, G4, and G5 in porcine rotaviruses.	J Clin Microbiol.	46(4)	1169-73	2008
Yan H, Koyano S, Inami Y, Yamamoto Y, Suzutani T, Mizuguchi M, Ushijima H, Kurane I, Inoue N.	Genetic variations in the gB, UL144 and UL149 genes of human cytomegalovirus strains collected from congenitally and postnatally infected Japanese children.	Arch Virol.	153(4)	667-74	2008
Khamrin P, Okitsu S, Ushijima H, Maneekarn N.	Novel nonstructural protein 4 genetic group in rotavirus of porcine origin.	Emerg Infect Dis,	14(4)	686-688	2008

Nguyen TA, Hoang L, Pham LD, Hoang KT, Okitsu S, Mizuguchi M, Ushijima H.	Norovirus and sapovirus infections among children with acute gastroenteritis in Ho Chi Minh City during 2005-2006.	J Trop Pediatr.	54(2)	102-13.	2008
Rathore A, Chatterjee A, Sood V, Khan SZ, Banerjea AC, Yamamoto N,. Dhole TN.	Risk for HIV-1 infection is not associated with repeat-region polymorphism in the DC-SIGN neck domain and novel genetic DC-SIGN variants among North Indians	Clin Chim Acta	391(1-2)	1-5	2008
Pham NTK, Trinh, QD, Khamrin P, Nguyen TA, Dey SK, Phan TG, Hoang LP, Maneenkarn N, Okitsu S, Ushijima H.	Sequence analysis of the capsid gene of Aichi viruses detected from Japan, Bangladesh, Thailand, and Vietnam.	J Med Virol	80(7)	1222-1227	2008
Rathore A, Chatterjee A, Sivarama P, Yamamoto N,. Singhal PK, Dhole TN	Association of RANTES _403 G/A, _28 C/G and In1.1 T/C polymorphism with HIV-1 transmission and progression among North Indians.	J Med Virol	80 (7)	1133 -1141	2008
Khamrin P, Maneekarn N, Peerakome S, Okitsu S, Mizuguchi M, Ushijima H.	Bovine Kobuviruses from Cattle with Diarrheic.	Emerg Infect Dis,	14(6)	985-986	2008
Schröder HC, Wang X, Tremel W, Ushijima H	Müller WE. Biofabrication of biosilica-glass by living organisms.	Nat Prod Res.	25(3)	455-474	2008
Dey SK, Islam A, Mizuguchi M, Okitsu S, Ushijima H.	Epidemiological and molecular analysis of astrovirus gastroenteritis in Dhaka City, Bangladesh.	J Trop Pediatr	54(6)	423-425	2008
Khamrin P, Peerakome S, Malasao R, Mizuguchi M, Okitsu S, Ushijima H, Maneekarn N.	Genetic characterization of group C rotavirus isolated from a child hospitalized with acute gastroenteritis in Chiang Mai, Thailand.	Virus Genes.	37	314-321	2008

Malasao R, Maneekarn N, Khamrin P, Pantip C, Tonusin S, Ushijima H, Peerakome S.	Genetic diversity of norovirus, sapovirus, and astrovirus isolated form children hospitalized with acute gastroenteritis in Chiang Mai, Thailand.	J Med Virol	80(10)	1749-1755	2008
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Yan H, Koyano S, Inami Y, Yamamoto Y, Suzutani T, Mizuguchi M, Ushijima H, Kurane I, Inoue N.	Genetic linkage among human cytomegalovirus glycoprotein N (gN) and gO genes, with evidence for recombination from congenitally and post-natally infected Japanese infants.	J Gen Virol	89(pt 9)	2275-2279	2008
Khamrin P, Maneekarn N, Peerakome S, Malasao R, Thongprachum A, Chan-It W, Mizuguchi M, Okitsu S, Ushijima H.	Molecular characterization of VP4, VP6, VP7, NSP4, and NSP5/6 genes identifies an unusual G3P[10] human rotavirus strain.	J Med Virol	81(1)	176-182	2008
Thongprachum A, Khamrin P, Saekhow P, Pantip C, Peerakome S, Ushijima H, Maneekarn N.	Analysis of the VP6 gene of human and porcine group A rotavirus strains with unusual subgroup specificities.	J Med Virol	81(1)	183-191	2008
Pham NT, Trinh QD, Nguyen TA, Dey SK, Phan TG, Hoang LP, Khamrin P, Maneekarn N, Okitsu S, Mizuguchi M, Ushijima H.	Development of genotype-specific primers for differentiation of genotypes A and B of Aichi viruses.	J Virol Method,	[Epub ahead of print]		2008

Kittigul L, Pombubpa K, Taweekate Y, Yeephoo T, Khamrin P, Ushijima H.	Molecular characterization of rotaviruses, noroviruses, sapovirus, and adenoviruses in patients with acute gastroenteritis in Thailand.	J Med Virol	81(2)	345-353.	2009
Dey SK, Hayakawa Y, Rhaman M, Islam R, Mizuguchi M, Okitsu S, Ushijima H.	G2 strain of rotavirus among infants and children, Bangladesh.	Emerg Infect Dis	15(1)	91-94.	2009
Takanashi S, Hashira S, Matsunaga T, Yoshida A, Shiota T, Phan TG, Khamrin P, Okitsu S, Mizuguchi M, Igarashi T, Ushijima H.	Dectection, genetic characterization, and quantification of Norovirus RNA from sera of children with gastroenteritis.	J Clin Virol	[Epub ahead of print]		2009
Khamrin P, Takanashi S, Chan-It W, Kobayashi M, Nishimura S, Katsumata N, Okitsu S, Maneekarn, N, Nishio O, Ushijima H.	Immunochromatograpy test for rapid detection of norovirus in fecal specimens.	J Virol Methods	[Epub ahead of print]		2009
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プロジェクト５：ウイルス（狂犬病ウイルス）					
Kojima, D., Chun-Ho, P., Yusuke, S., Inoue, S., Noguchi, A. and Toshifumi, O.	Pathology of the Spinal Cord of C57BL/6J Mice Infected with Rabies Virus (CVS-11 Strain).	J. V. M. S.	[in press]		2008

プロジェクト6：原虫（マラリア）

Saito-Nakano Y, Tanabe K, Kamei K, Iwagami M, Komaki-Yasuda K, Kawazu S, Kano S, Ohmae H, Endo T.	Genetic evidence for *Plasmodium falciparum* resistance to chloroquine and pyrimethamine in Indochina and the Western Pacific between 1984 and 1998.	*Am J Trop Med Hyg.*	79(4)	613 -619	2008
Izumiyama S, Omura M, Takasaki T, Ohmae H, Asahi H.	*Plasmodium falciparum*: development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer	*Exp Parasitol*	121(2)	144-150	2009
Nishimoto Y, Arisue N, Kawai S, Escalante A, Horii T, Tanabe K, Hashimoto T.	Evolution and phylogeny of the heterogeneous cytosolic SSU rRNA genes in the genus *Plasmodium.*	*Mol. Phylogenet. Evol.*	47	45-53	2008
Hayakawa T, Culleton R, Otani A, Horii T, Tanabe K.	Big bang in the evolution of extant malaria parasites.	*Mol. Biol. Evol.*	25	2233-2239	2008
Culleton R, Mita T, Ndounga M, Unger, P. Cravo, G. Paganotti, N. Takahashi, A. Kaneko, H. Eto, H. Tinto, C. Karema, U. D' Alessandro, V. do Rosário, T. Kobayakawa, F. Ntoumi, R. Carter, and K. Tanabe.	Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing.	Malaria J	7	174	2008