RESEARCH NOTE

PHYLOGENETIC RELATIONSHIP OF *PLASMODIUM FALCIPARUM* POPULATIONS IN THE PHILIPPINES

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Abstract. Malaria is one of the major infectious diseases in the Philippines. It is being targeted for control through sustained early diagnosis, treatment and mosquito control. It is in this light that understanding the genetic background of the parasite population is important not only for basic biology of the organism but also for epidemiology and control of the disease. In the present study, molecular phylogenetic relationships of the 3 *Plasmodium falciparum* populations in the Philippines with the other populations in the world were inferred based on polymorphisms of 9 highly polymorphic microsatellite DNA loci in the parasite genome. A total of 92 *P. falciparum* isolates collected from 3 provinces (Kalinga, Palawan and Davao del Norte) in the Philippines, and 8 from other populations (3 African, 2 South American, 2 Papua New Guinean, and 1 Thai) that were previously reported, were used for the analysis. The phylogenetic tree showed that the 3 Philippine populations were genetically divergent from each other as compared to the other populations. The branching pattern of the tree suggests that the 3 Philippine populations were relatively close to the Thai population, rather than the Papua New Guinean populations, indicating that the ancestor of the 3 Philippines populations were introduced from Indochina peninsula, and not from countries located south of the Philippines such as Papua New Guinea or Indonesia.

INTRODUCTION

Malaria is still one of the major health problems in the Philippines. It is being presently controlled through early diagnosis, treatment and mosquito control. These strategies are geared towards sustainability through social mobilization. Research studies are also being conducted to guide policy directions in the implementation of these primary control strategies. Along these lines, several epidemiological and molecular studies on drug resistant malaria in the country have been reported (Chen *et al*, 2003,2005; Hatabu *et al*, 2003; Rivera, 2005; Rivera *et al*, 2005). However, phylogenetic analysis of the parasites in this country has not been reported so far. Molecular phylogenetic analysis of a pathogen can tell us the population history of the organism, such as how the disease has spread from one endemic area to another or how a specific genotype (viz drug resistant genotype) has spread from one population to another.

Microsatellite DNA is a short tandem

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repeat sequence of a 2-6 bp unit, repeated 5-50 times and usually polymorphic in numbers of copies. It is generally considered that the influence of selective pressure on mutations in this sequence is very low in comparison with that on other sequences such as single nucleotide polymorphisms (SNPs). Therefore, microsatellite DNA marker is a highly powerful tool for analyzing population genetics, conservation biology, and forensic medicine. The genome of *P. falciparum* contains about 50% microsatellite DNA loci that are polymorphic, and microsatellite DNA is observed in every 20 kbp (on average) in the parasite genome (Anderson *et al*, 1999, 2000; Hartl *et al*, 2002).

In the present study, phylogenetic relationships of *Plasmodium falciparum* populations in the Philippines with other populations were inferred, based on analysis of microsatellite DNA polymorphisms in the parasite.

MATERIALS AND METHODS

The study areas, sample sizes (n), and years of the blood collection were as follows: Kalinga Province (northern part of Luzon Island), n = 22, collected in 2003 through 2005, Palawan Province (Palawan Island), n = 40, collected in 2003 through 2006, and at Davao Regional Hospital in Tagum City, Davao del Norte (southeastern part of Mindanao Island), *n* = 30, collected in 1999 through 2001 (Fig 1). These 3 study areas are sentinel provinces in the Philippines where malaria is endemic perennially, but do not represent all the *P. falciparum* endemic foci of the country.

From the 3 different study areas in the Philippines, a total of 92 *P. falciparum* isolates was collected from both symptomatic and asymptomatic malaria patients who visited health clinics or hospitals in the 3 provinces. Informed consents were obtained from all the patients prior to the blood collection. This study was performed according to the ethical guidelines for epidemiological studies provided by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Patient blood samples were frozen in liquid nitrogen tanks or preserved on filter papers (IsoCode Stix, Schleicher & Schuell, Germany) and kept at room temperature until examined. Parasite DNA was extracted from the frozen whole blood samples by phenol-chloroform after proteinase K digestion (Sambrook and Russell, 2001) or from the blood-spot samples on filter papers following the instruction manual.

Nine microsatellite loci were amplified by semi-nested PCR. They were as follows (chromosomal locations in parentheses): TA1 (chromosome 6), TA42 (chromosome 5), TA109 (chromosome 6), Poly α (chromosome 4), TA81 (chromosome 5), TA87 (chromosome 6), Pfg377 (chromosome 12), PfPK2 (chromosome 12), and 2490 (chromosome 10). PCR primer sets used and amplification conditions were according to the protocol of Anderson *et al* (1999), as these 9 loci are a subset of the 12 loci described by Anderson *et al* (1999) for analyzing characteristics of the population genetic structure of *P. falciparum* worldwide (Hartl *et al*, 2002; Hartl, 2004). Fluorescence-labeled PCR products were separated on Applied Biosystems Prism Genetic Analyzer 310 using Gene Scan version 3.1.2 with 500 ROX size standard (ABI, CA, USA).

The raw data obtained were the sizes (bp) of each PCR product that contained the microsatellite locus of *P. falciparum*. Different sizes of the PCR products amplified with the same PCR primer set were regarded as different alleles in the locus, because the size differences among different isolates were due to the different repeat numbers of the microsatellite locus. The data of the isolates that were found as multi-clonal infections were exclude in the analysis.

For inferring the phylogenetic relationships

of the *P. falciparum* populations in the Philippines with other populations, genetic distances between each two populations were calculated based on the allele frequencies of the 9 microsatellite loci of the parasite genome using D_A genetic distance (Takezaki and Nei, 1996; Nei and Kumar, 2000). The D_A was calculated for each pairwise comparisons as

$$D_A = \sum_{k=1}^{L} [1 - \sum_{i=1}^{qk} (x_{ik} y_{ik})^{1/2}]/L,$$

where *qk* is the number of the *k*th locus and L is the number of loci examined. The D_A distance is more effective for estimating the genetic distance between two populations based on the allele frequency data of microsatellite DNA markers than the Nei's standard genetic distance (Takezaki and Nei, 1996; Nei and Kumar, 2000). The allele frequencies data of 8 other *P. falciparum* populations (3 African, 2 South American, 2 Papua New Guinean and 1 Thai) reported previously (Anderson *et al*, 2000) were included in this analysis. A neighbor-joining tree (Saitou and Nei, 1987) was constructed based on the D_A distances using the computer program MEGA ver. 3 (Kumar *et al*, 2004).

RESULTS

Molecular phylogenetic relationships of the 3 *P. falciparum* populations in the Philippines with the other 8 populations are shown in Fig 2. The 3 Philippine populations were genetically highly divergent with each other in comparison with the other populations. This is attributed to the fact that the levels of the differences of the allele frequencies among the 3 Philippine populations were greater than those of the other populations.

DISCUSSION

It is known that allele frequencies of genes in a population can be changed through

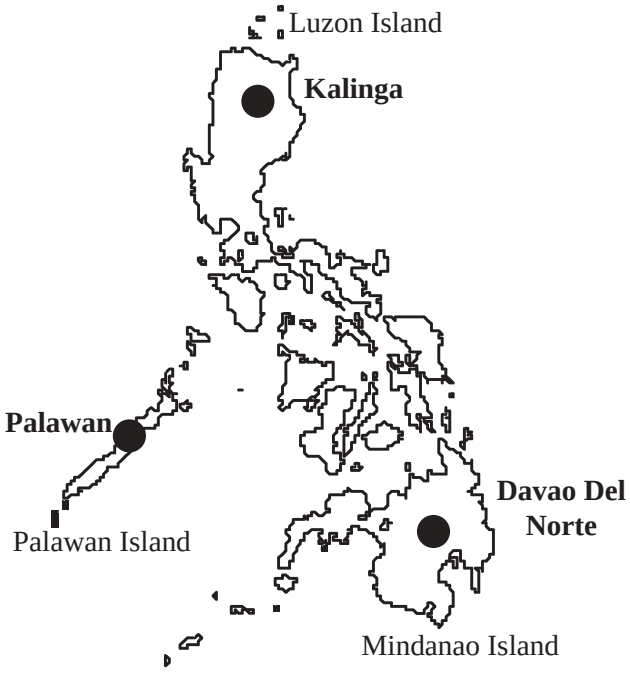

Fig 1–Study areas in the Philippines.

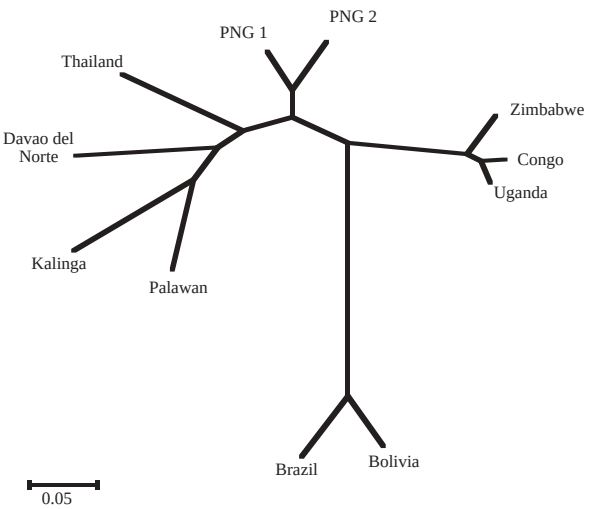

Fig 2–Phylogenetic tree of 3 *Plasmodium falciparum* populations from the Philippines and the 8 other populations. The phyllogenetic three was based on allele frequencies data of 9 microsatellite DNA sequences. PNG (Papua New Guinea) 1 and 2 are different parasite populations.

generations by many factors, such as selective pressure, random genetic drift, and migration (Graur and Li, 2000). For microsatellite DNA locus, random genetic drift is the most important factor that affects the allele frequency of the locus in the population. Microsatellite DNA is normally free from selective pressure as long as the locus is neither located on nor close to a gene that affects survival fitness of the individual. It is known that the effect of random genetic drift is greater when population size is smaller (Graur and Li, 2000). Therefore the relatively long branch lengths among the 3 Philippine populations can be attributed not only to their geographical isolation but also to their small population size.

Geographically, the southern part of the Philippines, such as Palawan Island and Mindanao Island, are close to Malaysian Borneo and Indonesia, where malaria is also endemic. Therefore, there is a possibility that *P. falciparum* in the southern part of the Philippines has been introduced from Malaysian Borneo and Indonesia by immigrants. However, the branching pattern of the tree shows that the 3 Philippine populations were divergent from a single common ancestor after diverging from the Thai population. This result suggests that the origin of the *P. falciparum* in the Philippines was from populations in the Indochina peninsula (Thailand), rather than from populations in Papua New Guinea. Another possibility is that the influence of introduction of *P. falciparum* from the countries located to the south of the Philippines (viz. Malaysian Borneo, Indonesia and Papua New Guinea) may be too small to affect the topology of the phylogenetic tree.

However, caution is needed in the interpretation of this phylogenetic tree, which is constructed based on the allele frequencies data of microsatellite DNA of *P. falciparum* because the mutation patterns of these loci have not fully understood. In general, the mutation pattern of microsatellite DNA loci is considered to be a stepwise mutation model in many organisms (Nei and Kumar, 2000). However, the 9 loci examined in this study showed patterns of variation that are inconsistent with the stepwise mutation model.

A mutation rate of microsatellite DNA of *P. falciparum* per generation calculated based on experimental crossing between 2 laboratory strains (Dd2 and HB3) has been estimated to be very fast (1.59×10^{-4} per generation) (Su *et al*, 1999). In the field, however, the time span for each generation of *P. falciparum* is expected to be different from one endemic area to anothers, being shorter in a high malaria transmission area than in a low malaria transmission area. Therefore, phylogenetic analysis based on microsatellite DNA polymorphisms of *P. falciparum* may show inaccurate phylogenetic relationships if parasite populations are collected from different levels of malaria transmission areas. Further study is required to estimate accurately the population history of the parasites.

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