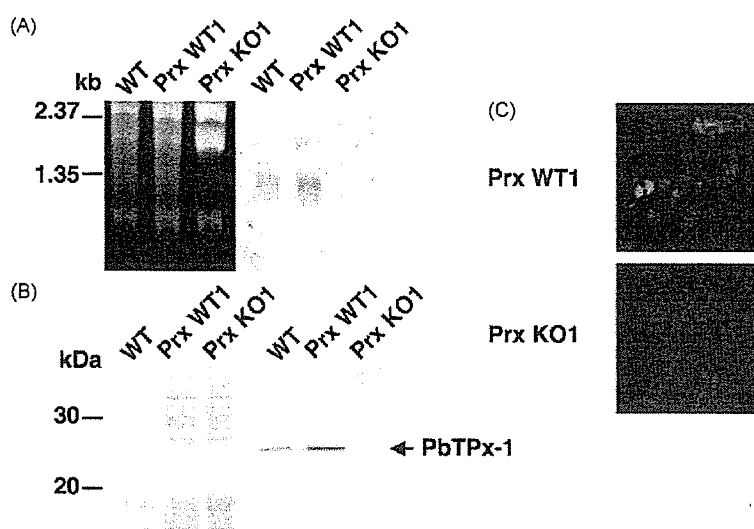

Fig. 2. Verification of the PbTPx-1 null phenotype in gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), Prx WT1 (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*), and Prx KO1 (*pbtpx-1* disruptant) were inoculated into mice, and parasite-infected erythrocytes, total protein, and total RNA of the parasite cells were prepared. (A) The absence of TPx-1-specific mRNA expression in Prx KO was examined by Northern blot analysis. Total RNA samples (20 μg) were separated on 1.5% agarose-formaldehyde gel (left panel), transferred to nylon membranes, and hybridized with probe (right panel). Ethidium bromide-stained gel showed equal loading. Molecular size markers in kb are indicated on the left. (B) The absence of TPx-1 protein in Prx KO was examined by Western blot analysis. Total protein samples (20 μg) were separated by SDS-PAGE (left panel) and probed with anti-TPx-1 antibody (right panel). Coomassie brilliant blue-stained gel showed equal loading. Protein size markers in kDa are indicated on the left. (C) The absence of TPx-1 protein in Prx KO was confirmed by indirect immunofluorescence assay. TPx-1 protein is stained in green, and parasite nuclei are stained in blue.

parasitemia reached approximately 4% (4–5 days after infection). Levels of 8-OHdG (ng/mg DNA) are expressed as the mean of triplicate measurements.

2.7. Statistical analysis

Differences were evaluated with Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Targeted disruption of the PbTPx-1 gene

Merozoites in segmented schizonts were transfected with the targeting construct by electroporation and were subsequently inoculated into naïve rats. Integration of the construct into the PbTPx-1 locus by homologous recombination resulted in disruption of the single-copy gene and insertion of a selectable marker, the DHFR-TS gene with a pyrimethamine-resistance mutation (*dhfr-ts/mt*), at the locus (Fig. 1A). Parasites with *pbtpx-1* disruption were selected by treatment with pyrimethamine. PCR and Southern blot analyses showed that parasites selected with pyrimethamine were a mixture of wild-type parasites and *pbtpx-1* disruptants (data not shown). Parasite populations were separated into two groups by limiting dilution and subsequent inoculation into 18–22 rats. In two independent electroporation experiments, six disruptant populations (Prx KO) were obtained and cloned. In the first experiment, three Prx KO populations (Prx KO1–3) and two wild-type parasite populations (Prx WT1 and 2) were obtained (Fig. 1B). In the second experiment, two Prx KO populations (Prx KO4 and 5) were obtained (data not shown). The *dhfr-ts* locus of Prx WT parasites was amplified by PCR and sequenced, and replacement with the pyrimethamine-resistance mutation was confirmed (data not shown). Northern blotting, Western blotting, and immunofluorescence assay confirmed the TPx-1-null phenotype of Prx KO1 (Fig. 2). This phenotype was also confirmed for all Prx KO populations (data not shown).

3.2. PbTPx-1 gene disruption does not affect asexual development

Prx KO1–3 and Prx WT1 and 2 were used to infect Balb/c mice, and the course of parasite development within erythrocytes was compared to that of the parent strain (WT) (Fig. 3A). Two Prx WT populations showed equal development and multiplication within erythrocytes, similar to WT, and they showed a high level of parasitemia (>5%) 5 days after infection. The courses of parasitemia observed in Prx KO1-infected mice were also similar to that in WT-infected mice. This phenotype was confirmed in Prx KO2 and 3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3B). There was no difference in the morphology of parasite cells between five Prx KO, two Prx WT, and WT populations (data not shown). These eight parasite populations did not kill mice until day 8 of observation. Prx KO1, Prx WT2, and WT populations each killed one of three mice, respectively, on day 9 of observation. It is known that Balb/c mice in some laboratories are resistant to the development of cerebral malaria (CM) induced by the *P. berghei* ANKA strain and do not develop lethal infection [25]. To determine the phenotype of *pbtpx-1* disruption in strains of mice with differing susceptibility to the *P. berghei*

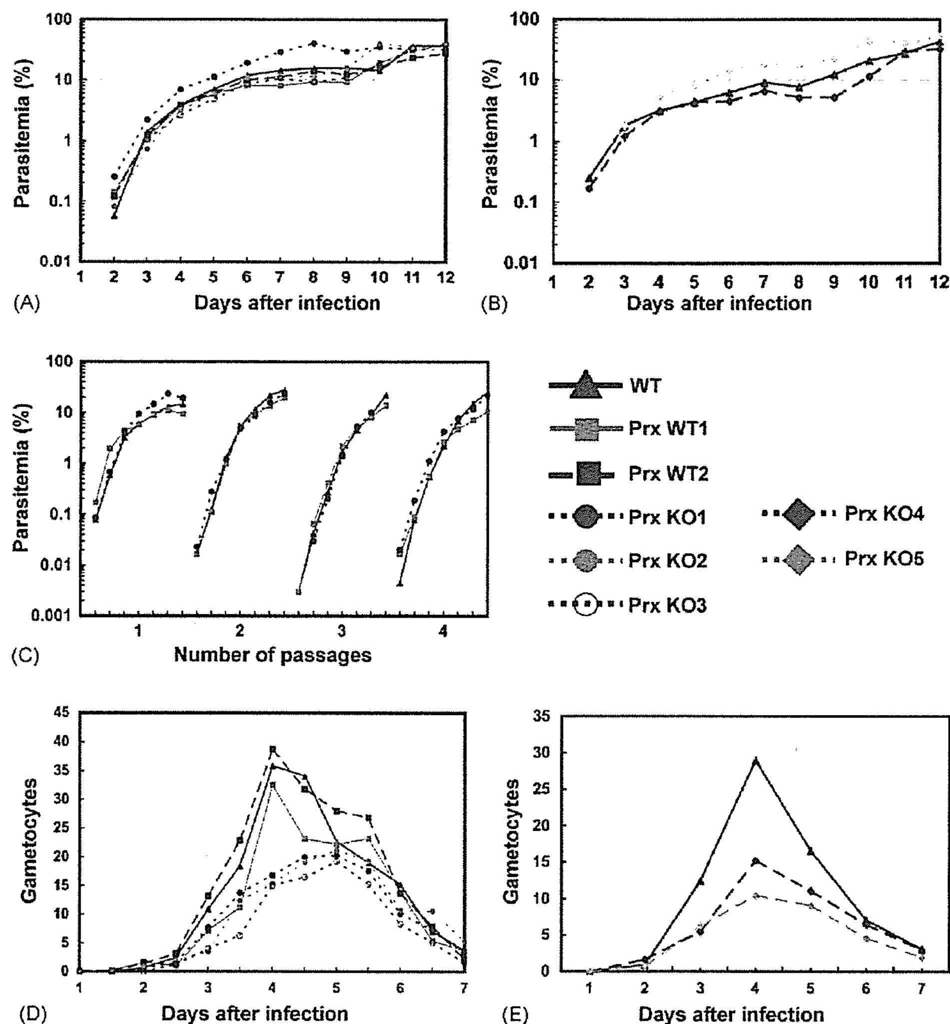

Fig. 3. Infection of mice with TPx-1 gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), two Prx WT (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*) and five Prx KO (*pbtpx-1* disruptant), were inoculated into mice, and the courses of parasite development in erythrocytes were observed. (A and B) Changes in parasitemia 12 days after infection (three mice per group: six groups in (A) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (B) WT and Prx KO4 and 5). (C) Changes in the course of parasitemia during four blood passages (two mice per group: three groups, WT, Prx WT1, and Prx KO1). (D and E) Changes in gametocytemia 7 days after infection (three mice per group: six groups in (D) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (E) WT and Prx KO4 and 5). Data are mean values of parasitemia percentage (A–C) and gametocyte number (gametocytes/10^4 erythrocytes) (D and E) in each group.

ANKA strain, C57B6 mice, which are known to develop CM and lethal infection [25], were infected with Prx KO1, and the course of parasite development within erythrocytes was compared to that of WT. Prx KO1 and WT multiplied equally and killed mice 7–8 days after infection. The morphology of parasite cells and the numbers of animals killed during 8 days of observation between the two parasite populations did not differ (data not shown).

A function of 2-Cys Prx in the prevention of damage to DNA by oxidative stress has been suggested in mammals [26]. Therefore, the cumulative effect of oxidative stress on parasite growth in vivo in the *pbtpx-1* disruptant was evaluated. For this purpose, Prx KO1, Prx WT1, and WT were continuously maintained in Balb/c mice by inoculating parasite-infected blood and comparing the course of parasitemia between passages. Blood passage was performed on the 7 day of infection and was repeated four times. The courses of parasitemia observed in Prx KO1-, Prx WT1-, and WT-infected mice from the first to the fourth passage are shown in Fig. 3C. The course of parasitemia after four passages compared to that after the first inoculation did not differ between the three parasite populations. Passage was repeated six more times, and the course of parasitemia observed for the three parasite populations at the 10th passage was similar to that observed at the fourth passage (data not shown). To determine whether *pbtpx-1* disruption enhances DNA damage in the parasite genome, the level of 8-OHdG, a marker of oxidative DNA damage, was compared between Prx KO1 and WT. The level of 8-OHdG in the parasite genome was similar between Prx KO1 (11.7 ng/mg DNA) and WT (9.1 ng/mg DNA), suggesting that *pbtpx-1* disruption does not affect the physiologic level of DNA oxidation. This result was confirmed in two independent experiments (data not shown).

3.3. *PbTPx-1 gene disruption affects gametocytogenesis*

To evaluate the effect of *pbtpx-1* disruption on development of sexual-stage parasites, the numbers of gametocytes in parasite-infected blood were counted and compared among Prx KO1–3, Prx WT1 and 2, and WT (Fig. 3D). In Prx WT1- and WT-infected mice, gametocytes were observed from 2 days after infection; they increased in number with parasitemia progression and peaked 4 days after infection. Gametocytemia, recorded as the number of gametocytes/10^4 erythrocytes in Prx WT1- and WT-infected mice at the day 4 of infection, was 32.5 ± 5.5 and 35.8 ± 11.6, respectively ($P = 0.34$). The course of gametocytemia between Prx WT1- and WT-infected mice did not differ. This phenotype was confirmed in two populations of Prx WT. In Prx KO1-infected mice, gametocytes were also observed from 2 days after infection and peaked 5 days after infection. The peak of gametocytemia in Prx KO1-infected mice was thus delayed 1 day from that of Prx WT1- and WT-infected mice. Gametocytemia, recorded in Prx KO1-infected mice as the number of gametocytes/10^4 erythrocytes on day 5 of infection, was 20.2 ± 0.9. The highest number of gametocytes was thus significantly lower ($P < 0.01$) in Prx KO1-infected mice than in WT-infected mice. The *pbtpx-1* disruption phenotype, in addition to the delay in gametocytemia peak, showed a significant decrease in the numbers of gametocytes ($P < 0.02$) in Prx KO1–3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3E).

To determine whether *pbtpx-1* disruption affects the male/female ratio of gametocytes or exflagellation activity of male gametocytes, Prx KO1, Prx KO4, Prx WT1, and WT were used to infect mice (Table 1). The male/female ratios of gametocytes in the three parasite populations at the day of peak gametocytemia were similar: 0.44, 0.53, and 0.64 for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 0.62 and 0.50 for Prx KO4- and WT-infected mice in experiment 2. This phenotype was confirmed in five populations of Prx KO (data not shown). The percentages of exflagellation, calculated according to the numbers of exflagellation centers and the numbers of male gametocytes in the blood at the day of peak gametocytemia, were 70, 64, and 76% for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 59 and 60% for Prx KO4- and WT-infected mice in experiment 2. Thus, the majority of male gametocytes observed in blood were equally viable in terms of exflagellation activity among the three parasite populations. This finding suggests that low gametocytemia in Prx KO is attributed to impaired development of gametocytes rather than to their decreased survival.

4. Discussion

The results presented here suggest that *pbtpx-1* disruption does not affect asexual intraerythrocytic growth of parasites. The results from passage experiments and evaluation of 8-OHdG suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx [26], and that it is not essential for asexual parasite growth in mouse erythrocytes. However, the fact that the parasite lacks catalase and genuine GSH peroxidase and that the major cytosolic peroxide-detoxifying capacity appears to be provided by Prx may contradict the present findings [1,2]. Microarray analysis of the disruptant and the parent parasite may provide insights with respect to redundancy in parasite antioxidant defenses and compensation for the lack of Prx. Our results may not indicate that the gene is inessential for asexual growth in *P. falciparum* because there are differences in the life cycle of the mammalian stage between *P. falciparum* and rodent malaria parasites. *P. falciparum* develops in erythrocytes sequestered in the microvasculature, where the parasite may experience more severe oxidative stress than do parasites in the blood circulation [5]. The parasite may require Prx for development under such stressful conditions. Such a possibility should be investigated in a monkey model of *P. falciparum* infection [27]. The relatively high level of 8-OHdG formation in the parasite genome compared to that in mammalian cells [24] may reflect the absence of nuclear Prx in this organism [1,2,9]. How malaria parasites deal with the accumulation of oxidative DNA damage is of interest; a hypothetical protein with putative excision DNA repair function or DNA glycosylase function (PF10835c) has been identified in the genome of the malaria parasite.

Table 1
Male/female ratios of gametocytes and exflagellation activity of male gametocytes in TPx-1gene-disrupted *P. beghei*[a]

	Experiment 1			Experiment 2	
	WT	Prx WT1	Prx KO1	WT	Prx KO4
Peak of gametocytemia					
Gametocyte number[b]	30 ± 5.5	37 ± 5.5	18 ± 2.5	31 ± 3.2	14 ± 1.6
Days after inoculation	4.0	4.0	4.5	4.0	4.5
Male/female ratio					
Day of peak gametocytemia	0.64	0.53	0.44	0.50	0.62
Day 5.5	1.06	1.07	0.92	1.01	1.15
Exflagellation (%)[c]					
Day of peak gametocytemia	76 (52–88)	64 (51–81)	70 (53–81)	60 (54–64)	59 (55–63)
Day 5.5	16 (10–20)	13 (7–27)	24 (9–54)	14 (12–17)	4.1 (0.6–10)

[a] Values are means ± standard deviations (gametocyte number), mean (male/femal ratio), and mean with range (exflagellation, %) calculated from data obtained from four mice.

[b] Gametocytes per 10^4 erythrocytes.

[c] Exflagellation (%) = (number of exflagellation centers in 10^4 erythrocytes/number of male gametocytes in 10^4 erythrocytes) × 100.

Nonetheless, the results presented here suggest that PbTPx-1 is required for normal gametocyte development; however, it does not affect the male/female ratio of gametocytes. We could not observe the defect in gametocyte development in *P. falciparum*, since we disrupted the gene in the FCR-3 strain, which produces only a little numbers of gametocyte [16]. Gametocytes are sexual-stage parasites involved in the transition between the mammalian host and the mosquito. This stage arises during asexual cycling, and it has generally been accepted that the trophozoites of the preceding asexual generation are already committed to either develop into gametocytes or continue asexual cycling [28]. Although the mechanism by which PbTPx-1 contributes to gametocyte development is unknown, ROS and antioxidants, including Prx, are known to influence the expression of a number of genes and to influence signal transduction pathways during cell differentiation and during organismal development [29,30]. A model in which Prx regulates peroxide-mediated signaling cascades by acting as a floodgate for H_2O_2 in mammalian cells has been proposed [12,14]. Homology modeling of PbTPx-1 based on structural alignment with human homologues showed that it possesses a redox-sensitive type of peroxidatic active site structure, which enables the enzyme to act according to the floodgate model (data not shown). A model in which 2-Cys Prx regulates activation of stress-activated mitogen-activated protein kinase (MAPK) (Styl) by forming peroxide-induced disulphide complexes in yeast has also been proposed [31]. A molecule homologous to Styl (MAPK1) has been identified in the *P. falciparum* genome, although its function is unknown. Gametocyte development can be induced by environmental factors such as host factors or drug treatments, and there is consistent evidence for the involvement of signal transduction pathways in this process [28]. PbTPx-1 may be involved in transducing extracellular signals for gene expression and in initiation of gametocyte development. If this is the case, gametocyte development and its sexual determination may occur independently, with PbTPx-1 participating only in the former process. Alternatively, PbTPx-1 may contribute to protein synthesis in both male and female gametocyte development as a molecular chaperone. Evidence that Prx acts as a molecular chaperone in yeast and in human cells has recently been reported [32,33]. PbTPx-1 may not take part in the process of male gamete formation, including gender-specific signaling, because the disruptant retained normal exflagellation activity [34,35]. Whether it participates in female gametogenesis will require fertilization experiments in vitro and in the mosquitos [35,36]. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, the present study suggests the involvement of this moleclue in the sexual development of the malaria parasite. Further studies to elucidate the role of TPx-1 in gametocyte development will provide further insight into the involvement of this antioxidant protein in the sexual development of malaria parasites and may provide novel transmission-blocking strategies.

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Reemerging vivax malaria: changing patterns of annual incidence and control programs in the Republic of Korea

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Abstract: Changing patterns of the reemerging *Plasmodium vivax* malaria in the Republic of Korea (South Korea) during the period 1993 to 2005 are briefly analyzed with emphasis on the control measures used and the effects of meteorological and entomological factors. Data were obtained from the Communicable Diseases Monthly Reports published by the Korea Center for Disease Control and Prevention, and webpages of World Health Organization and United Nations. Meteorological data of Kangwon-do (Province) were obtained from local weather stations. After its first reemergence in 1993, the prevalence of malaria increased exponentially, peaking in 2000, and then decreased. In total, 21,419 cases were reported between 1993 and 2005 in South Korea. In North Korea, a total of 916,225 cases were reported between 1999 and 2004. The occurrence of malaria in high risk areas of South Korea was significantly ($P < 0.05$) correlated with the mosquito population but not with temperature and rainfall. Control programs, including early case detection and treatment, mass chemoprophylaxis of soldiers, and international financial aids to North Korea for malaria control have been instituted. The situation of the reemerging vivax malaria in the Republic of Korea is remarkably improving during the recent years, at least in part, due to the control activities undertaken in South and North Korea.

Key words: *Plasmodium vivax*, vivax malaria, reemerging malaria, incidence, geographical distribution, seasonality, South Korea, North Korea

INTRODUCTION

Vivax malaria due to *Plasmodium vivax*, the only naturally occurring human malaria species in the Republic of Korea (hereafter referred to as South Korea), was endemic in South Korea until the late 1970s, when the country became malaria free (Paik et al., 1988; Chai, 1999). In particular, during the Korean War (1950-1953), 15,000 South Korean soldiers and more than 3,000 U.S. soldiers were reported to have contracted vivax malaria (Jones et al., 1953; Chai,

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1999). Subsequently, malaria cases rapidly decreased, with the implementation of the National Malaria Eradication Service, which was established jointly by the South Korean government and the World Health Organization (WHO) in 1959 (Ministry of Health and Social Affairs, Republic of Korea, 1966). Consequently, after the 1970s, indigenous malaria cases were almost unheard of, though 2 such cases were reported in 1984 (Soh et al., 1985). During the same period a substantial number of imported malaria cases was reported (Chai, 2002).

However, indigenous vivax malaria reemerged in 1993; a South Korean soldier working at the western edge of the demilitarized zone (DMZ; the border between South and North Korea) in Kyonggi-do (Province), was confirmed to have contracted *P. vivax* malaria (Chai et al., 1994). Thereafter, the number of malaria cases increased exponentially year by year, peaking in 2000 (Feighner et al., 1998; Chai, 1999; Lee et al., 2002; Park et al., 2003). North Korea, which stated that it was free of malaria from the 1970s, also started reporting cases in 1998 from the northern part of the DMZ bordering South Korea (Chol et al., 2005). Since then, the number of malaria cases in North Korea has increased dramatically and reached around 300,000 in 2001. Therefore, in 1999, the North Korean government developed a national malaria control program in cooperation with WHO, to reduce the malaria burden (Chol et al., 2005).

In South Korea, during the period 1993-1996, the outbreak area was confined to the northern part of Kyonggi-do and northwestern Kangwon-do, near the DMZ (Chai, 1999). However, after 1997, the outbreak area extended in an easterly direction to the northeastern region of Kangwon-do and in a southerly direction in Kyonggi-do (Lee et al., 2002; Park et al., 2003), and it was feared that this southward trend would continue. To cope with this risk, the present national malaria control program was launched in 1997 (Korea Center for Disease Control and Prevention, Republic of Korea, 2002). This program includes early case detection and treatment, chemoprophylaxis of soldiers, vector control, personal protection, and financial aids to North Korea for malaria control.

In addition to control activities, meteorological (temperature and rainfall) and entomological factors (mosquito density) may have significant impacts on malaria transmission. For instance, the incidences of malaria were related to local climatic variables in China (Bi et al., 2003) and Rwanda (Loevinsohn, 1994). However, in South Korea, no published data is available concerning the relations between temperature, rainfall, the population density of the vector mosquitoes, and the incidence of malaria.

The aim of the present paper is to briefly summarize vivax malaria outbreaks over the period 1993 to 2005 in South Korea, and to analyze the efficacies of the control activities implemented since 1997, and the impacts of meteorological and entomological factors on disease occurrence.

MATERIALS AND METHODS

Malaria is designated an important communicable disease and case details must be reported immediately to the Ministry of Health and Welfare in South Korea. In the present study, all cases reported since the first reemergence of indigenous vivax malaria case in 1993 were subjected to analysis. Patients' occupations, i.e., civilians, soldiers on duty, and retired soldiers, were obtained from the Communicable Diseases Information System (http://dis.cdc.go.kr) and from the Communicable Diseases Monthly Reports issued during the study period by the Korea Center for Disease Control and Prevention (KCDC), Ministry of Health and Welfare, South Korea. Information about malaria prevalence in North Korea and financial support for malaria control was obtained from the KCDC, World Health Organization (WHO) (http://www.who.int) and the United Nations Office for the Co-ordination of Humanitarian Affairs (OCHA), Pyongyang, Democratic Peoples' Republic (DPR) of Korea (http://www.humanitarianinfo.org/dprk) and from the United Nations (UN) (http://www.reliefweb.int).

The annual geographic distributions of malaria cases in South Korea over 12 years were determined by grouping cases by city and province where patients were located when a diagnosis of malaria was

made. Information about the time required to make a diagnosis of malaria after the onset of symptoms, were obtained from the reports of patients admitted to local health centers and hospitals in Kangwon-do, South Korea.

Meteorological data, i.e., mean temperature and rainfall for the main transmission period (the 6 mo period from May to October), recorded at local weather stations in Cheolwon-gun, Kangwon-do, a malaria endemic area near the DMZ, were obtained from the Korean Meteorological Administration, South Korea.

The population densities of adult anopheline mosquitoes, over 90% of which is *Anopheles sinensis*, the main vector mosquito for vivax malaria in the Republic of Korea (Chai, 1999), were determined during the transmission period at one location in Cheolwon-gun, Kangwon-do, from 1993 to 2004 by two (WS Seok and YS Kim) of the authors. Adult anopheline mosquitoes emerged from the first week of May (1-10 mosquitoes/trap/night) and disappeared from the last week of October (0-14 mosquitoes/trap/night). A black light trap (Nozawa type, Shinyoung Korea Co., Seoul, Korea) was hung from a fence about 1.5 m above the ground in shed housing one cow. Black light traps were operated without additional attractants from 18:00 to 06:00 hr twice a week during the study period. All captured mosquitoes were transported the following morning to the Kangwon Institute of Health and Environment, where they were identified, separated, and the number of anopheline mosquitoes was counted.

Mass chemoprophylaxis (1 chloroquine tablet; 300 mg base) has been administered by the Ministry of Defense to a total of 985,282 soldiers working around outbreak areas (northern parts of Kyonggi-do and Kangwon-do) weekly from 1997 to 2005. Chemoprophylaxis was also prescribed to a total of 12,189 US soldiers in South Korea during the period 1997-2000. Retiring Korean soldiers were advised to take primaquine 15 mg base daily for 14 days for chemoprophylaxis against the liver stage parasite at the time of their retirement.

Spearman's correlation analysis was used to examine correlations between the number of new malaria cases, year, climatic factors, i.e. annual mean temperature (°C) and rainfall (mm), and the annual mean number of mosquitoes trapped during May to October. The monthly mean number of anopheline mosquitoes, and the mean number of mosquitoes trapped weekly and annual totals were calculated from mean monthly numbers trapped during the 6 month transmission period. P values of < 0.05 were regarded as statistically significant.

RESULTS

During the past 13 years (1993-2005), at least 937,634 indigenous vivax malaria cases have been reported in the Korean peninsula (South Korea and North Korea) (Table 1). Based on available data between 1999 and 2004 in South and North Korea, the number of cases reported peaked in 2001 with 298,058 cases in the Korean peninsula. In South Korea, during the period 1993-2005, a total of 21,419 indigenous vivax malaria and 488 imported malaria cases were confirmed (Table 1). The indigenous malaria patients included 8,353 (39.0%) civilians and 13,066 (61.0%) soldiers, including 5,626 retired soldiers (26.3%) who had retired from military service for less than one year at disease onset. The number of reported cases peaked in 2000 with 8.9 cases per 100,000 of the South Korean population. Thereafter, the number of reported cases declined sharply by approximately 26-40% per annum to 1.8-2.9 cases per 100,000 of the population in 2004-2005 (Table 1).

The annual incidence rate (including retired soldiers discharged < 1 year prior to onset and soldiers on duty) peaked at 457.3 cases per 100,000 soldiers in 2000. The incidence decreased by more than 84% between 2000 and 2004, but then increased by 35% in 2005 (Table 1). The same trend, i.e., peak in 2000 followed by a sharp decline until 2004 and a rise in 2005, was observed both among serving and retired soldiers. Among civilians, the annual incidence rate peaked at 3.3-3.4 cases per 100,000 in 1999-2000, and then decreased to 0.9 in 2004, but increased again to 1.9 in 2005 (Table 1).

In 1999, total 95,960 malaria cases were reported in

Table 1. Vivax malaria cases reported annually among civilians and soldiers in South Korea and North Korea

	Number of reported cases (Annual cumulative incidence per 100,000 population)													
Group	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	Total
South Korea														
Civilians	0	2	7	46	361	1,148	1,541	1,580	1,047	864	542	413	802	8,353
	(0.0)	(0.0)	(0.0)	(0.1)	(0.8)	(2.5)	(3.3)	(3.4)	(2.3)	(1.9)	(1.2)	(0.9)	(1.9)	
Soldiers														
Retired[a)]	0	1	12	25	207	1,127	996	1,273	756	472	279	159	319	5,626
On duty	1	18	88	287	1,155	1,655	1,085	1,288	685	430	282	236	230	7,440
Subtotal	1	19	100	312	1,362	2,782	2,081	2,561	1,441	902	561	395	549	13,066
	(0.2)	(3.4)	(17.9)	(55.7)	(243.2)	(496.8)	(371.6)	(457.3)	(257.3)	(161.1)	(102.0)	(71.8)	(96.7)	
Total	1	21	107	358	1,723	3,930	3,622	4,141	2,488	1,766	1,103	808	1,351	21,419
	(0.0)	(0.0)	(0.2)	(0.7)	(3.6)	(8.3)	(7.7)	(8.9)	(5.4)	(3.9)	(2.4)	(1.8)	(2.9)	
North Korea[b)]	ND[e)]	ND	ND	ND	ND	ND	95,960	204,428	295,570	240,339	46,251	33,677	ND	916,225
							(432.3)	(920.8)	(1,331.4)	(1,082.6)	(208.3)	(151.7)		
Total, indigenous cases							99,582	208,569	298,058	242,105	47,354	34,485		937,634
US Army soldiers[c)]	0	1	0	14	34	47	53	42	29	41	23	15	ND	299
Imported malaria[d)]	ND	6	30	41	40	63	53	41	43	44	61	37	29	488

[a)]Retired soldiers, who were infected during military service in risk areas and developed febrile illness at home after discharge from the service.
[b)]Data were obtained from webpages of World Health Organization (http://www.who.int), the United Nations Office for the Co-ordination of Humanitarian Affairs, Pyongyang, Democratic Peoples' Republic of Korea (http://www.humanitarianinfo.org/dprk), and from the United Nations (http://www.reliefweb.int).
[c)]United States Army cases were diagnosed either in South Korea or after return to the United States.
[d)]Imported malaria cases in South Korea, who were infected in Southeast Asia, Africa, Oceania, and in Central and South Americas.
[e)]ND = no available data.

North Korea, but this increased explosively 3-folds between 1999 and 2001 (1,331.4 per 100,000 North Korean population), and after 2002 decreased sharply to 208.2 and 151.7 per 100,000 population in 2003 and 2004, respectively (Table 1).

The annual numbers of malaria cases reported by nationwide administrative districts (provinces and large cities) are given in Fig. 1, as sums of 2 years, from 1994-1995 to 2004-2005. Of the total 21,419 cases registered during the 12 year period, most (85.4%) developed febrile illness in northern provinces and cities near the DMZ (the highest risk areas), including 10,411 cases (48.6%) in Kyonggi-do, 3,083 (14.4%) in Kangwon-do, 2,710 (12.7%) in suburban Seoul, and 2,089 (9.8%) in suburban Incheon (Fig. 1). In Kyonggi-do, the most serious outbreak area, the peak incidence occurred in 1998 with 2,197 cases, and decreased gradually afterwards. However, in Kangwon-do, the second most serious outbreak area, the peak incidence of 825 new cases, occurred in 2000. Small numbers of cases were reported from various Provinces and Cities countrywide through 12 years, although these cases were predominantly among retired soldiers who had served in northern parts of Kyonggi-do or Kangwon-do about a year previously, thus indicating a long incubation period. The numbers of patients reported in other Provinces and Cities are shown in Fig. 1.

Meteorological data, i.e., annual mean temperature and rainfall, and mean mosquito population densities, during 1993-2004, were analyzed in terms of their relationships with the annual total numbers of malaria cases reported in Kangwon-do, South Korea (Table 2). Spearman's correlation analysis showed that the occurrence of malaria in high risk areas was correlat-

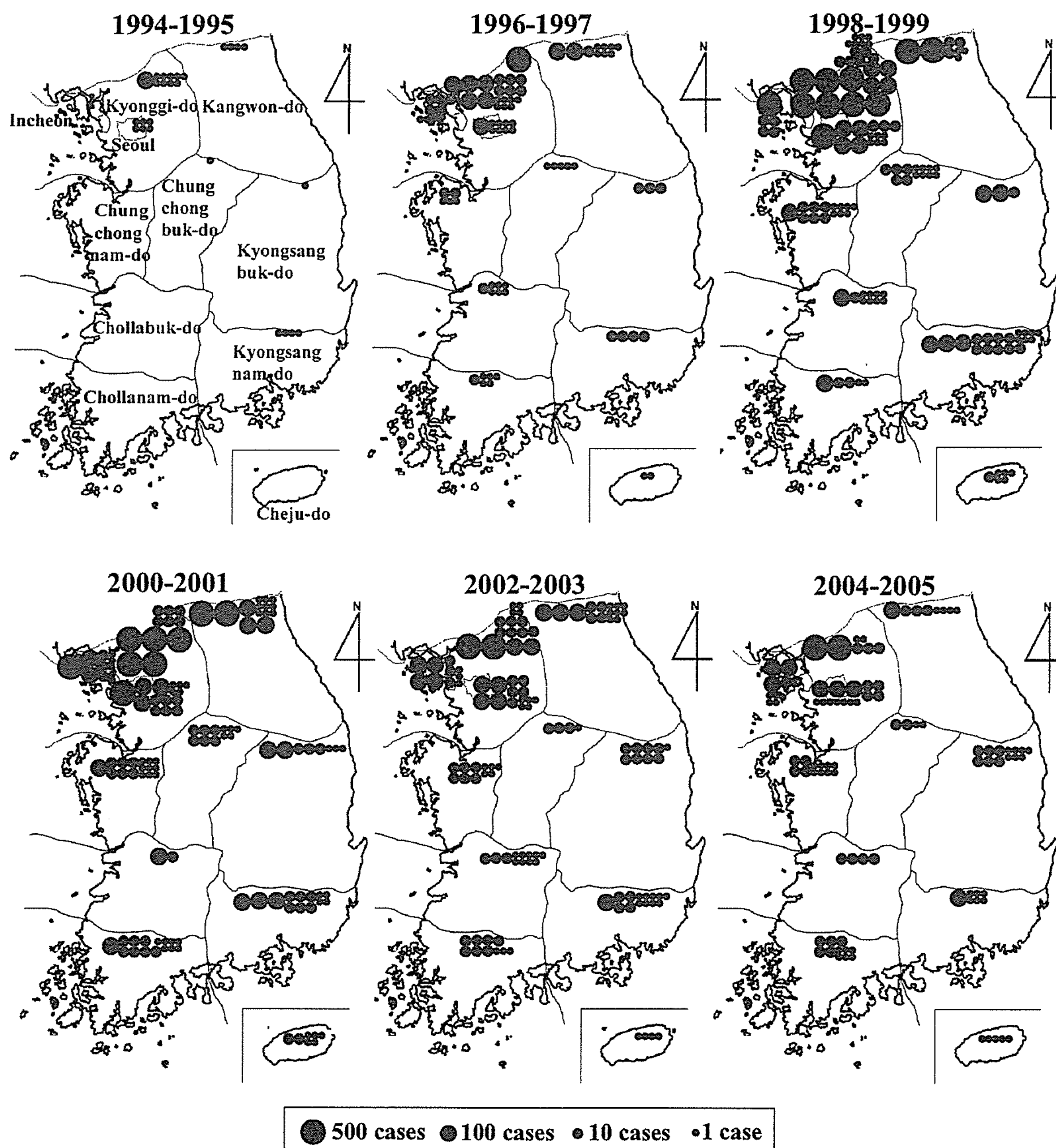

Fig. 1. Maps of South Korea, showing the numbers of indigenous vivax malaria cases reported by administrative districts (Provinces and Cities, including Incheon and Seoul) from 1994-1995 to 2004-2005. The figure represents the number of patients who developed febrile illness and were diagnosed in the district, but does not necessarily mean actual contraction of malaria in each district.

ed with the mosquito population, only with low significance ($P = 0.048$), and no positive association was observed with temperature or rainfall (Table 2).

The time required for a diagnosis of malaria from the onset of febrile paroxysm has reduced year by year in most outbreak areas of Kangwon-do. For

Table 2. Mean annual temperatures, rainfalls, and anopheline mosquito population densities compared to annual malaria incidence rate in Kangwon-do, South Korea, from 1993 to 2004

Item	Mean annual variables												*P*-value[a)]
	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	
Mean temperature (°C)[b)] (May - Oct.)	18.0	19.6	18.5	18.8	18.8	19.8	19.6	19.5	19.9	18.3	18.3	18.7	0.096
Mean rainfall (mm) (May - Oct.)	130.0	165.8	229.3	196.3	162.9	225.6	255.4	162.9	195.9	165.9	243.4	162.9	0.593
Mean number of mosquitoes[c)] (May - Oct.)	604	799	779	290	550	1,293	1,801	1,574	890	1,194	1,282	1,299	0.048
Annual number of patients (Malaria incidence; I)[d)]	0 (0.0)	0 (0.0)	4 (0.3)	40 (2.6)	177 (11.5)	519 (33.4)	514 (33.0)	825 (53.1)	544 (35.0)	216 (14.0)	132 (8.6)	43 (2.8)	-

[a)]*P*-value: between the total number of patients and climatic variances (mean temperature and mean rainfall) and mean number of mosquitoes.
[b)]Main transmission season in each year.
[c)]Mean number of anopheline mosquitoes/cow/trap/night. Over 90% were *Anopheles sinensis*, the main vector mosquito.
[d)]Incidence (I) per 100,000 population in Kangwon-do, South Korea. The correlation coefficient between I and mean temperature was 0.605, between I and rainfall 0.514, and between I and mosquito density 0.591.

example, 66 (44.0%) of 150 patients were diagnosed and treated within 6 days of symptom onset in 1999, but this increased to 61.7-73.6% during 2000-2002 (Table 3).

In 2001, the South and North Korean governments started to provide budgetary supports for facilitating malaria control programs in both countries. In the case of South Korea, 2 northern provinces (Kyonggi-do and Kangwon-do) and one city (Incheon), received budgetary supports for mosquito control from the KCDC, and this was followed by fiscal support from provincial and city health bureaus from 2001 to 2004. The total expenditures over this 4 year period in South Korea was 5,154,700 USD (Table 5). In North Korea, during the period 2001-2004, international supports for malaria control have been provided by WHO (for education and assistance for technician training), the International Federation of Red Cross and Red Crescent (IFRC) (anti-malarial drugs) and South Korea. The total amount of anti-malarial aid given to North Korea over this 4 year period was 3,150,650 USD (Table 5). In South and North Korea, during the same period (2001-2004), 8,305,350 USD were spent on malaria control. Items of supports provided by South Korea to North Korea included anti-malarial drugs (chloroquine and primaquine), mosquito control related materials and equipments (insecticide impregnated-bednets, personal protection fabrics, insecticides, and insecticide spraying equipment) and laboratory supplies for prompt diagnosis (microscopes and staining reagents) in local health centers and hospitals, and small amount of cash for the education of health personnel (Table 5).

DISCUSSION

Our study demonstrated that the number of reemerging vivax malaria cases in South Korea increased exponentially during the years 1993-2000, but then decreased steadily until 2004 with a slight increase in 2005. This post 2000 decrease in malaria incidence was observed countrywide, and included high risk areas near the DMZ. Control programs were operated, including mass chemoprophylaxis, vector control, and financial aids to North Korea for malaria control, and are believed, at least in part, to have contributed to the reduction of malaria incidence.

Malaria transmission requires the combined presence of the *Plasmodium* parasite, the anopheline mosquito vector, and the human host. Both parasites and vectors are strongly affected by climate, for example, temperature determines parasite and vector develop-

ment, and rainfall provides the water required for vector breeding. In Rwanda and China, monthly mean temperature was found to play an important role in malaria transmission (Loevinsohn, 1994; Bi et al., 2003). However, with regard to rainfall levels, reports are contradictory; some studies have reported that rainfall is a key factor (Lindblade et al., 1999; Bi et al., 2003), whereas others have reported negative effects (Singh and Sharma, 2002). In a previous South Korean study, increases in temperature and precipitation were found to be correlated with seasonal vector mosquito population densities, and with the subsequent seasonal incidence of malaria (Lee et al., 2002). In this previous study, 2 climatic factors were compared with averaged data collected over a 30-year period, though no statistical analysis was performed (Lee et al., 2002).

In our study, low grade statistical significance (P = 0.048) was observed in the correlations between anopheline mosquito densities and the annual malaria incidence during the 1993-2004 period in Kangwon-do, but none between the climatic variables and malaria incidence. Although mosquito densities during 1998-2001 could not be clearly correlated with malaria incidences, mosquito densities during these years were significantly ($P < 0.05$) higher than those before 1998 when malaria incidence was comparatively low (Table 2). Nevertheless, detailed ecological and epidemiological studies are needed to assess the true impact of climatic variables on malaria outbreaks in South Korea.

Regardless of the control strategy adopted, the early diagnosis and proper treatment of those infected is essential (Lee et al., 2003). In South Korea, the average duration between the onset of malarial fever and diagnosis at a health center or a hospital was 23.6 days in 1995, 9.5 days in 1997, and 8.0 days in 2000 (Park et al., 2003). Since 2000 about two-thirds of malaria patients have been more quickly diagnosed and treated, within 6 days, for example, in Kangwon-do (Table 3). Moreover, in 2004, the average detection time became as short as 3-4 days in highly endemic areas in Kangwon-do (data not shown), and it is speculated that early case detection substantially reduced the malaria transmission from patients to mosquitoes.

Table 3. Days required for confirmation of malaria diagnosis after the onset of symptoms among civilians and veterans in risk areas of Kangwon-do, South Korea, from 1999 to 2002

Year	Number of cases (%)				
	Days until diagnosis after the onset of febrile paroxysm				
	0-6	7-15	16-25	> 26	Total number of cases
1999	66 (44.0)	53 (35.3)	18 (12.0)	13 (8.7)	150
2000	209 (73.6)	66 (23.2)	6 (2.1)	3 (1.1)	284
2001	138 (66.7)	51 (24.6)	12 (5.8)	6 (2.9)	207
2002	58 (61.7)	21 (22.3)	11 (11.7)	4 (4.3)	94

Mass chemoprophylaxis is another major contributor to the observed recent reduction in malaria cases. Before 1997, more than 80% of malaria cases occurred in northern parts of Kyonggi-do and Kangwon-do, the major outbreak areas (Chai, 1999; Park et al., 2003), and most patients were soldiers stationed near the DMZ. Therefore, mass chemoprophylaxis was administered to soldiers located in these endemic areas in 1997 and has continued ever since (Table 4). From 1997 to 2005, a total of 985,282 soldiers received chloroquine and primaquine prophylaxis. As a consequence, malaria incidence among soldiers on duty and retired soldiers decreased rapidly during 2001-2005. This prophylaxis program must have been largely responsible of the observed reduction in the malaria incidence.

However, it should be noted that the proportion of civilian cases among all malaria cases has increased from 38.2% in 2000 to 50.6% in 2004. This increase in the proportion of civilian cases suggests an increase in local transmission away from the DMZ (civilians usually live some distance from the DMZ). This increase in local transmission is also suggested by the fact that outbreak areas have expanded in southerly and easterly directions since 1998 (Chai, 1999; Park et al., 2003; Yeom et al., 2005).

It is also of note that a substantial number of cases

Table 4. Chemoprophylaxis of military soldiers of South Korea, US Army, and North Korea

	Number of soldiers									
	1997	1998	1999	2000	2001	2002	2003	2004	2005	Total
ROK Army[a)]	15,981	37,529	61,772	90,000	90,000	140,000	160,000	190,000	200,000	985,282
US Army	35	2,485	8,510	1,159	ND[c)]	ND	ND	ND	ND	12,189
North Korea[b)]	0	0	0	0	100,000	350,000	300,000	300,000	ND	1,050,000
Total	16,016	40,014	70,282	91,159	190,000	490,000	300,000	300,000	200,000	2,047,471

[a)]Republic of Korea Army.
[b)]Figures are based on the amount of anti-malarial drugs used for chemoprophylaxis and treatment supported by South Korea.
[c)]ND = no available data.

Table 5. Financial support for malaria control in South Korea and North Korea

Group/Year		Expenditures (in USD)				
		2001	2002	2003	2004	Total
South Korea	Mosquito control[a)]	689,100	734,900	816,600	2,914,100	5,154,700
North Korea[†]	Supported by South Korea					
	Anti-malarial drugs, mosquito control, etc.[b)]	490,000	620,000	700,000	700,000	2,510,000
	Education[c)]	38,450	26,900	26,900	26,900	119,150
	Supported by IFRC[d)]					
	Anti-malarial drugs, etc.	21,000	21,000	158,000	321,500	521,500
	Subtotal	549,450	667,900	884,900	1,048,400	3,150,650
Total		1,238,550	1,402,800	1,701,500	3,962,500	8,305,350

[a)]For insecticide purchase and equipments purchase for insecticide spraying in Kyonggi-do, Kangwon-do, and Incheon city, South Korea.
[b)]Anti-malarial drugs included chloroquine and primaquine (for treatment of 100,000-300,000 patients per year), and mosquito control included nsecticides like permethrin, devices for insecticide spraying, and insecticide-treated bed nets and clothes. Others included lancets, pH meters, staining reagents for blood smears and microscopes. Data are from World Health Organization, (WHO) and Korea Center for Disease Control and Prevention, South Korea.
[c)]For training laboratory technicians, entomologists, and health workers (total 70 persons per year) to help build a sustainable national ability to control malaria by WHO.
[d)]International Federation of Red Cross and Red Crescent Societies.

(more than 30% of all patients during the period 1998-2004) have been reported in Pusan, Taegu, Kyongsangbuk-do, and Kyongsangnam-do regions, which are considerably removed from major outbreak areas. Such cases may include retired soldiers, travelers to major outbreak areas, and locally infected civilians. In the case of retired soldiers living in these areas, the majority were probably infected while working in major outbreak areas, and developed febrile illness after a long incubation period of 5-13 mo (Chai, 1999), whereas travelers may have developed fever after a short (within 1 mo) or a long incubation period. It is unfortunate that no study has yet reported firm evidence of local malaria transmission in areas remote from the major outbreak areas.

There is no doubt that vivax malaria reemergence in South Korea was originally caused by infected mosquitoes originating from North Korea and the DMZ (Chai, 1999; Park et al., 2003). In this regard, it is worth mentioning that genotypes of circumsporozoite protein (Kho et al., 1999), merozoite surface protein (MSP)-1 (Zakeri et al., 2003), Duffy-binding protein

(Kho et al., 2001), apical membrane protein antigen-1 (Han et al., 2002), and MSP-3α (Han et al., 2004) of the reemerging vivax malaria in South Korea are similar to those found in the North Korean (NK) strain. Infected mosquitoes probably constantly migrate from North to South Korea (Cho et al., 2002), and we suggest that a large proportion of malaria cases in South Korea have resulted from this influx. Moreover, it is evident that the malaria situation in northern South Korea will be influenced by that in North Korea.

With regard to the malaria situation in North Korea, no data was available before 1997. However, recently some occurrence data has become available (Global Funds to Fight AIDS, Tuberculosis and Malaria, 2003; United Nations, 2003; United Nations Office for the Co-ordination of Humanitarian Affairs DPRK, 2003, 2004; World Health Organization, 2004; Chol et al., 2005). Indigenous cases have now been reported from 1997 (Chol et al., 2005), and nationwide patient numbers increased sharply prior to 2001, but then dramatically reduced to 2004. Several factors may have facilitated the increase in malaria cases during 1999-2001 in North Korea. Such factors may include changes in agricultural practices, such as, reduced use of pesticides and changes in rice field irrigation, intermittent big flooding, increased vector host densities, and inadequate health care delivery system.

However, a malaria control program was implemented in endemic areas of North Korea, in South and North Hwanghae-do (Provinces) during 2001-2003 by the National Program Office of WHO, in North Korea (Chol et al., 2005). It has been stated that the prevalence of malaria began to decline immediately after implementing this control program, and individual awareness regarding malaria increased rapidly. However, more precise data are required to better assess the situation of vivax malaria in North Korea.

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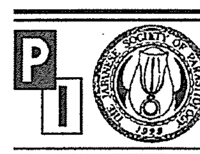
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Genetic diversity and kinetic properties of *Trypanosoma cruzi* dihydroorotate dehydrogenase isoforms☆

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Abstract

Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme in the de novo pyrimidine biosynthetic pathway and is essential in *Trypanosoma cruzi*, the parasitic protist causing Chagas' disease. *T. cruzi* and human DHOD have different biochemical properties, including the electron acceptor capacities and cellular localization, suggesting that *T. cruzi* DHOD may be a potential chemotherapeutic target against Chagas' disease. Here, we report nucleotide sequence polymorphisms of *T. cruzi DHOD* genes and the kinetic properties of the recombinant enzymes. *T. cruzi* Tulahuen strain possesses three *DHOD*genes: *DHOD1* and *DHOD2*, involved in the pyrimidine biosynthetic (*pyr*) gene cluster on an 800 and a 1000 kb chromosomal DNA, respectively, and *DHOD3*, located on an 800 kb DNA. The open reading frames of all three *DHOD* genes are comprised of 942 bp, and encode proteins of 314 amino acids. The three *DHOD* genes differ by 26 nucleotides, resulting in replacement of 8 amino acid residues. In contrast, all residues critical for constituting the active site are conserved among the three proteins. Recombinant *T. cruzi* DHOD1 and DHOD2 expressed in *E. coli* possess similar enzymatic properties, including optimal pH, optimal temperature, V_{max}, and K_m for dihydroorotate and fumarate. In contrast, DHOD3 had a higher V_{max} and K_m for both substrates. Orotate competitively inhibited all three DHOD enzymes to a comparable level. These results suggest that, despite their genetic variations, kinetic properties of the three *T. cruzi*DHODs are conserved. Our findings facilitate further exploitation of *T. cruzi* DHOD inhibitors, as chemotherapeutic agents against Chagas' disease.
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Keywords: *Trypanosoma cruzi*; Dihydroorotate dehydrogenase; Pyrimidine–biosynthetic gene cluster; Genetic diversity; Drug target

1. Introduction

Chagas' disease is caused by infection with the parasitic protist, *Trypanosoma cruzi*. This disease affects about 17 million people in Latin America, with about 25% of the population estimated to be at risk [1]. Medication is usually effective when given during the acute phase, whereas, during the chronic phase, infected tissues become gradually impaired and the disease is no longer curable [2]. The chemotherapeutic drugs currently used are highly toxic and often lead to discontinuation of the therapy [1]. Thus, development of new drugs with low toxicity is needed.

Pyrimidine biosynthesis is an essential biological activity, which is conducted by both de novo and salvage pathways. The de novo pathway is comprised of six sequential enzymatic reactions. Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme in the pathway catalyzing the oxidation of dihydroorotate (DHO) to orotate. Human DHOD, which is localized in the inner membrane of mitochondria, uses ubiquinone as electron acceptor [3,4]. In contrast, *T. cruzi* DHOD is a cytosolic protein that preferentially uses fumarate as electron acceptor, producing succinate [5]. Phylogenetic reconstruction of DHOD has shown that *T. cruzi* and human DHOD have the different origins, belonging to families 1A and 2, respectively

Abbreviations: DHOD, dihydroorotate dehydrogenase; ACT, aspartate carbamoyltransferase; *pyr*, de novo pyrimidine biosynthetic gene.

☆ Sequence availability: The sequences reported in this paper for the *Trypanosoma cruzi* dihydroorotate dehydrogenase (DHOD) gene loci *DHOD1*, *DHOD2*, and *DHOD3* have been placed into the GenBank, EMBL, and DDBJ databases under the accession numbers AB212955, AB212956, and AB212957, respectively.

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[6]. In conjunction with the unique properties of *T. cruzi* DHOD, we have previously reported that marine algae extracts inhibit the *T. cruzi* DHOD activity in a non-competitive manner, as well as inhibiting the growth and infection of *T. cruzi* in mammalian cells [7]. Thus, inhibitor(s) specific for *T. cruzi* DHOD may be promising candidate drugs in the treatment of Chagas' disease.

In *T. cruzi*, the *DHOD* gene is localized in the de novo pyrimidine biosynthetic (*pyr*) gene cluster, which contains five genes that encode all six enzymes in the de novo pyrimidine synthesis pathway [8]. Two complete sets of the *pyr* gene cluster have been found in all *T. cruzi* strains to date, along with amplification of parts of the cluster or individual *pyr* genes [9]. For example, three *DHOD* gene loci have been identified in the *T. cruzi* Tulahuen strain, two localized to the *pyr* gene clusters on an 800 and a 1000 kb chromosomal DNA. The third *DHOD* locus clusters only with the gene encoding the second enzyme in the pathway, aspartate carbamoyltransferase (ACT), on an 800 kb DNA (see Fig. 1). In addition, we observed extensive nucleotide and amino acid sequence polymorphisms among the *ACT* gene loci, suggesting that *T. cruzi DHOD* gene, as well as *ACT*, is polymorphic [9].

The presence of multiple *DHOD* gene loci and their possible genetic variation may indicate, however, that the encoded enzymes would not present drug targets. That is, a drug may inhibit one DHOD enzyme but not others, thus allowing parasite survival. Nevertheless, we have recently demonstrated that targeted disruption of at least two *DHOD* gene loci in *T. cruzi* Tulahuen resulted in loss of viability of the parasite even in the presence of pyrimidine substrates of the salvage pathway, suggesting that *T. cruzi* DHOD promises to be a drug target [10].

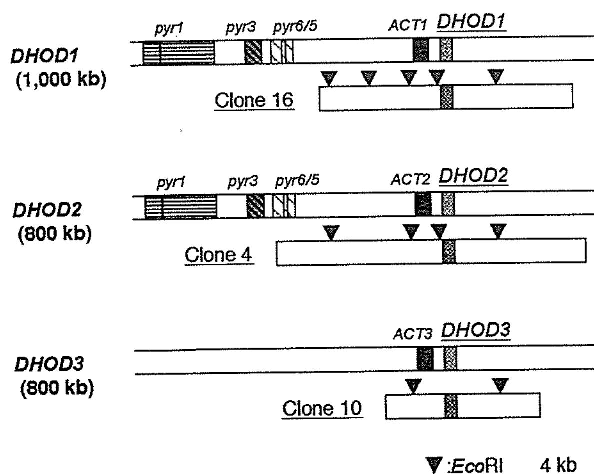

Fig. 1. Schematic illustration of the three dihydroorotate dehydrogenase (*DHOD*) gene loci in *Trypanosoma cruzi* Tulahuen strain. *DHOD1* is localized in the pyrimidine–biosynthetic (*pyr*) gene cluster on a 1000 kb chromosomal DNA. This cluster contains five genes of the de novo pyrimidine biosynthetic pathway: *pyr1*, encoding carbamoyl–phosphate synthetase II; *pyr3*, encoding dihydroorotase; *pyr6/5*, encoding orotidine-5′-monophosphate decarboxylase-orotate phosphoribosyltransferase; *ACT*, encoding aspartate carbamoyltransferase, and *DHOD*. *DHOD2* is localized in the *pyr* gene cluster on an 800 kb DNA. *DHOD3* is present on an 800 kb DNA and linked only to the *ACT3* gene. *DHOD1*, *DHODH2*, and *DHOD3* were isolated from phage clones, Nos. 16, 4, and 10 [9].

In the present study, we determined whether *T. cruzi DHOD* genes are polymorphic and, if so, whether polymorphisms would affect their enzymatic properties. We observed extensive nucleotide substitutions among the three *DHOD* loci, with replacement of 26 out of 945 nucleotides (2.75%), resulting in substitutions of 8 amino acids. Nevertheless, kinetic constants of the three recombinant DHODs were comparable. Our findings provide insight into *T. cruzi* DHOD as a drug target in the treatment of Chagas' disease.

2. Materials and methods

2.1. Nucleotide sequence determination of the DHOD loci in T. cruzi Tulahuen

We selected three phage clones, Nos. 16, 4, and 10, representing *DHOD1*, *DHOD2*, and *DHOD3*, respectively, that have been screened from a λEMBL3 genomic DNA library of *T. cruzi* Tulahuen strain [9]. *DHOD1* is present in the *pyr* gene cluster on a 1000 kb chromosomal DNA; *DHOD2* is in the *pyr* gene cluster on an 800 kb DNA; and *DHOD3* clusters partially with *ACT* gene on an 800 DNA (Fig. 1). Each phage DNA was digested with *Eco*RI, and the resulting DNA fragment, carrying a *DHOD* gene, was subcloned in the *Eco*RI site of pUC18. The open reading frames of *DHOD1*, *DHOD2*, and *DHOD3* were completely sequenced using synthetic oligonucleotide primers and an automated DNA sequencer (Model CEQ8000, Beckman Coulter Inc., Fullerton, CA, USA) under the conditions recommended by the manufacturer.

2.2. Bacterial expression of the recombinant T. cruzi DHODs

The open reading frame of each *DHOD* was PCR amplified using sense primers specific for *DHOD1* (5′-CACCATGATGCGTCTGAAACTCAA-3′), *DHOD2* (5′-CACCATGATGTGTCTGAAGCTCAA-3′), and *DHOD3* (5′-CACCATGACGTGTCTGAAGCTCAA-3′), a common antisense primer (5′-TCACTCAATTGTCTTGACAC-3′), and KOD plus DNA polymerase (High-fidelity type, Toyobo Co., Ltd., Osaka, Japan). The PCR products were cloned in pET100/D-TOPO® vector (Invitrogen, San Diego, CA, USA), which allows N-terminal fusion of a His_6-tag with the recombinant protein. Each cloned *DHOD* gene was sequenced and confirmed to be free of PCR-generated errors. The recombinant plasmids were used to transform BL21-CodonPlus® (DE3)-RP competent *E. coli* (Invitrogen), and expression of the recombinant *T. cruzi* DHOD was carried out as described, with minor modifications [7]. Briefly, expression was induced by incubating the cells in 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries, Tokyo) for 3 h. The cytosolic fraction of the bacteria was loaded onto a His•Bind® resin column (Novagen, EMD Biosciences, Inc., Madison, WI, USA) and the bound, His_6-tagged DHOD was eluted with 0.5M NaCl/0.5M imidazole/20 mM Tris, pH 7.4. This buffer in the protein eluate was

exchanged with phosphate-buffered saline (PBS, pH 7.2) using a PD-10 column (Amersham Biosciences Corp., Piscataway, NJ, USA). The final DHOD preparations were stored at − 80 °C until use.

2.3. DHOD assays

DHOD activity was determined by incubating the enzyme in the presence of dihydroorotate (substrate) and fumarate (electron acceptor) in 50 mM potassium phosphate, and measuring orotate production by absorption at 300 nm ($\varepsilon = 3.30\ mM^{-1}\ cm^{-1}$). Optimal pH of each enzyme was determined by altering the pH of the potassium phosphate from 6.0 to 8.0. For kinetic analyses, each 100 μl reaction mixture, containing recombinant DHOD, dihydroorotate, fumarate, and 50 mM potassium phosphate, pH 7.0, was pre-incubated at 37 °C for 5 min and the reaction was started by addition of DHO. To evaluate orotate inhibition, the reaction mixture was incubated with orotate for 5 min before starting the reaction.

```
DHOD1     ATGATGCGTCTGAAACTCAACCTCCTCGACCATGTGTTCGCCAACCCCTTCATGAACGCC   60
DHOD2     ------T-------G---------------------------------------------
DHOD3     ----C-T-------G---------------------------------------------
AA rep        M->T
                C->R

DHOD1     GCGGGTGTTCTCTGCAGCACCGAGGAAGACCTGCGCTGCATGACAGCCTCCTCCAGCGGC  120
DHOD2     -----A------------------------------------------------------
DHOD3     ------------------------------------------------------------
AA rep

DHOD1     GCACTTGTGTCGAAGAGCTGCACGAGTGCACCTCGCGATGGTAACCCCGAGCCGCGTTAC  180
DHOD2     ------------------------------------------------------------
DHOD3     ------------------------------------------------------------
AA rep

DHOD1     ATGGCCGTTCCACTTGGGAGCATCAATTCTATGGGGCTGCCTAACCTGGGCTTTGATTTC  240
DHOD2     ------T----------A------------------------------------------
DHOD3     -----------------A------------------------------------------
AA rep          F->V

DHOD1     TATTTGAAATACGCCATCGATCTGCACGATTACAGCAAGAAGCCGCTTTTTCTCTCCATT  300
DHOD2     ----------------G-------------------------------------------
DHOD3     ----------------G------------------------A---------G--------
AA rep                    S->I                                L->V

DHOD1     TCAGGTCTTTCCGTGGAGGAGAATGTGGCGATGGTGCGCCGCCTTGCCCCTGTGGCGCAG  360
DHOD2     ------------------------------------------------------------
DHOD3     ------------------------------------------------------------
AA rep

DHOD1     GAAAAAGGTGTATTGTTGGAGTTGAATCTTTCCTGCCCGAATGTGCCCGGCAAACCGCAG  420
DHOD2     ------------------------------------------------------------
DHOD3     ------------------------------------------------------------
AA rep

DHOD1     GTGGCCTATGACTTTGAGGCGATGCGCACTTACTTGCAGCAGGTGTCGTTGGCATACGGA  480
DHOD2     --------------------------------------------------------T---
DHOD3     --------------------------------------------------------T---
AA rep

DHOD1     TTGCCCTTTGGGGTGAAGATGCCGCCGTACTTTGATATTGCACACTTTGATACGGCTGCT  540
DHOD2     C----------C--------------------C---------------------------
DHOD3     C-C--------C--------------------C---------------------------
AA rep

DHOD1     GCTGTCTTGAATGAGTTCCCACTTGTCAAGTTTGTGACGTGTGTGAACAGTGTCGGCAAC  600
DHOD2     --------A--------------------------------------T------------
DHOD3     --------A--------------------------------------T------------
AA rep

DHOD1     GGCCTTGTTATTGATGCGGAGAGTGAGTCTGTTGTCATCAAACCCAAACAAGGGTTTGGT  660
DHOD2     ------------------------------------------------------------
DHOD3     ------------------------------------------------------------
AA rep

DHOD1     GGTATTGGCGGCAAGTATATCCTCCCTACAGCGCTGGCGAACGTGAATGCATTCTACCGC  720
DHOD2     ---C----------------------------AT-A------------------------
DHOD3     ---C----------------------------AT-A------------------------
AA rep       L->I

DHOD1     CGTTGTCCGGATAAGTTGGTCTTTGGCTGCGGCGGCGTTTACAGCGGCGAGGATGCCTTC  780
DHOD2     --C--C------------------------------------------------------
DHOD3     --C--C------------------------------------------------------
AA rep

DHOD1     TTGCATATACTTGCCGGTGCGTCGATGGTGCAAGTGGGAACTGCACTGCAGGAGGAGGGC  840
DHOD2     -----------------------------------------------------------G
DHOD3     -----------------------------------------------------------G
AA rep

DHOD1     CCCGGCATTTTTACGCGTCTTGAAGATGAGTTGCTGGAGATCATGGCACGGAAGGGGTAC  900
DHOD2     -----------------------------------------------G------------
DHOD3     -------------G---------------------------------G------------
AA rep                 T->R

DHOD1     AAGACTCTGGAGGAGTTCCGTGGACGTGTCAAGACAATTGAGTGA                945
DHOD2     -G-------------------------------------------
DHOD3     ---------------------------------------------
AA rep     R->K
```

Fig. 2. Nucleotide sequences of the three *Trypanosoma cruzi* DHOD genes. Substitutions in the *DHOD2* and *DHOD3* nucleotide and deduced amino acid sequences are shown relative to those of *DHOD1*. Dashes indicate identical nucleotides.

3. Results

3.1. Nucleotide sequence polymorphism in the *T. cruzi DHOD* gene

To determine whether the three *DHOD* gene loci in *T. cruzi* Tulahuen strain are polymorphic, we subcloned the inserts of three *DHOD*-carrying phage clones, encoding DHOD1 (clone 16), DHOD2(clone 4), and DHOD3(clone 10) (Fig. 1). Each clone was found to have an open reading frame of 942 bp, without deletions or insertions, encoding 314 amino acids. We found that the three *DHOD* loci differed by 26 of the 942 nucleotides (2.75%), leading to replacement of 8 amino acids. Four of these amino acid replacements were not equivalent (Fig. 2). We calculated the molecular mass of DHOD1, DHO2, and DHOD3 as 34.2, 34.2, and 34.1 kDa, respectively, and their isoelectric points (pIs) were pH 5.6, 5.4, and 5.6, respectively.

We also found that the rate of nucleotide substitutions differed between each pair of *DHOD* gene loci. *DHOD1* differs from *DHOD2* and *DHOD3* by 21 (2.22%) and 23 (2.43%) nucleotides, respectively, resulting in 5 and 6 amino acid replacements, respectively. In contrast, *DHOD2* and *DHOD3* were found to differ by only 8 nucleotides (0.84%), resulting in replacement of 5 amino acids.

T. cruzi DHOD is classified as a family 1A enzyme [6], and, using *Lactococcus lactis* DHODA, the amino acid residues participating in the catalytic reaction have been well characterized [11–13]. The corresponding amino acids, Lys45, Asn69, Leu73, Asn129, Cys132, Asn134, Ser197 and Asn196, were all conserved among the three forms of *T. cruzi* DHOD.

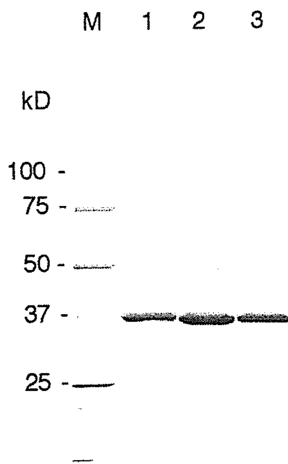

Fig. 3. Expression and purification of the recombinant *Trypanosoma cruzi* DHODs. Following expression in *E. coli* as His_6-tagged recombinant proteins and purification, 1 μg of purified DHOD1, DHOD2, and DHOD3 were loaded onto lanes 1–3, respectively, of a 10% SDS-polyacrylamide gel under denaturing conditions. Molecular weights of the protein standards (lane M) are indicated in kilodaltons (kD).

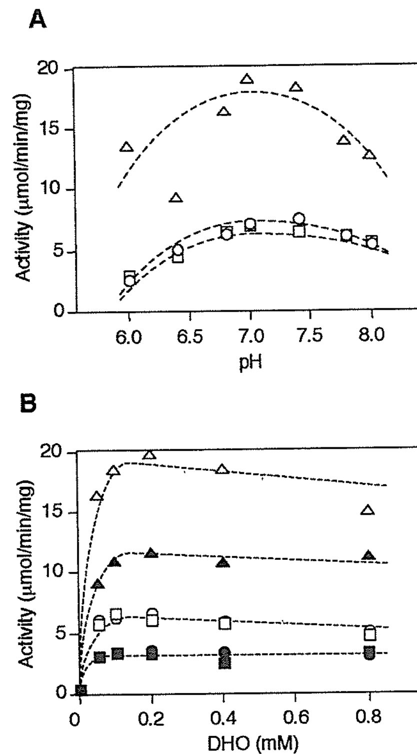

Fig. 4. Effects of pH (A) and temperature (B) on the activity of recombinant *Trypanosoma cruzi* DHODs. (A) Activity of DHOD1 (circle), DHOD2 (square), and DHOD3 (triangle) in the presence of 1 mM dihydroorotate (DHO) and 1 mM fumarate at 37 °C. (B). Activity in the presence of 50–800 μM DHO and 1 mM fumarate at 37 °C (open characters) and 25 °C (closed).

3.2. Expression of the recombinant *T. cruzi* DHODs

To determine the effect of the amino acid replacements, we assayed the kinetic properties of the three recombinant *T. cruzi* DHODs. Each recombinant *T. cruzi* DHOD with an N-terminal His_6-tag was expressed and purified by affinity chromatography as a single 37 kDa band (Fig. 3). Each of the purified DHODs was yellow, with absorption spectra having maxima at 375 and 459 nm, suggesting the presence of a flavin nucleotide, such as FMN, as a prosthetic group (data not shown).

Prior to kinetic analyses, we determined the optimal pH and temperature of each *T. cruzi* DHOD. We found that the optimal pH for all three was pH 7.0 (Fig. 4A). As *T. cruzi* parasitizes insects and mammals, we then measured the DHOD activity at 25 °C for the insect stage and at 37 °C for the mammalian

Table 1
Comparison of K_m values of *T. cruzi* DHODs

Enzyme	DHO (μM)[a]	Fumarate (μM)[b]
DHOD (native)[c]	25.9±5	53.4±3
DHOD1	30±3	30±8
DHOD2	30±3	35±6
DHOD[c]	24.3±4	43.9±2
DHOD3	71±6	67±3

In the presence of 1 mM fumarate[a] or 1 mM DHO[b].
[c]At 25°C [5].
All results are reported as mean±SD.

Table 2
Inhibition constants (K_i) for orotate against the recombinant *T. cruzi*

Enzyme	DHO (μM)[a]	Fumarate (μM)[b]
DHOD1	57.3±6.9	62.2±1
DHOD2	71.4±8.2	77.3±21.4
DHOD2[c]	20.5±3.6	15.6±4.32
DHOD3	42.4±17.4	49.0±17.8

In the presence of 1 mM fumarate[a] or 1 mM DHO[b].
[c]At 25°C [5].
All results are reported as mean±SD.

stage. All three *T. cruzi* DHODs showed a higher V_{max} at 37 °C than at 25 °C (Fig. 4B). These results can be correlated with findings that amastigotes replicating in mammalian cells depend on de novo synthesis of pyrimidines and are likely to require their accelerated biosynthesis [14]. Thus, all subsequent kinetic analyses of the *T. cruzi* DHODs were carried out at pH 7.0 and 37 °C.

We found that DHOD3 had the highest V_{max} at both 25 and 37 °C (Fig. 4B). This was unexpected, since none of the critical amino acids constituting the active site of the enzyme had been replaced.

3.3. Kinetic properties of the recombinant *T. cruzi* DHODs

We have previously demonstrated that native DHOD purified from epimastigotes of *T. cruzi* Tulahuen and recombinant DHOD (DHOD2 without the His_6-tag) shared very similar kinetic properties [5]. Since the kinetic properties of recombinant DHOD2 are similar in the presence or absence of the His_6-tag (Tables 1 and 2), we analyzed the kinetics of the three DHODs with His_6-tag. We found that the K_m values of DHOD1 and DHOD2 were similar and consistent with those reported earlier (Table 1). In contrast, DHOD3 showed higher K_m values for both DHO and fumarate.

Orotate is a competitive inhibitor for family 1A DHOD, including *T. cruzi* DHOD [5]. We found that orotate competitively inhibited the activity of all three DHODs with DHO and fumarate at comparable levels (Table 2). In contrast, the K_i values were slightly higher than those reported previously, which may have been due to differences in the assay temperature (Table 2).

4. Discussion

Variations in the number of *DHOD* gene copies among *T. cruzi* strains and in the enzymatic properties of proteins expressed from the different loci may lead to difficulty in developing clinically useful DHOD-specific inhibitors. In the present study, we demonstrated genetic polymorphisms in the three *T. cruzi DHOD* gene loci and the kinetic profiles of the recombinant DHODs. We observed replacement of 2.75% of the nucleotides, a rate much higher than that of other *T. cruzi* genes, such as dihydrolipoamide dehydrogenase (0.2%), and regulatory-particle non-ATPase subunit 1 of the proteasome (0.8%) [13,15]. Recent whole-genome sequencing of *T. cruzi* CL Brener, a reference strain of *T. cruzi* genome project, clearly revealed the presence of three complete *pyr* gene clusters and one partial cluster including *ACT* and *DHOD* in its genome [16,17]. Analysis of the putative four *DHOD* genes in the CL Brener genome showed replacement of 3.5% of the nucleotides, consistent with our findings (data not shown). In addition, when the intraspecies variations of the dihydrofolate reductase–thymidylate synthase (1473 bp) gene were analyzed using 18 strains of *T. cruzi*, it was found that 25 (1.7%) of 1473 nucleotides were replaced, resulting in three amino acid substitutions [18]. In contrast, the rate of nucleotide substitutions in the *ACT* gene, which juxtaposed to all three *DHOD* gene loci in *T. cruzi* Tulahuen, is 3.0% [9]. Thus, it is likely that the rate of evolution of the *pyr* genes, including *ACT* and *DHOD*, is faster than that of other genes, despite the essential role of the former in de novo pyrimidine biosynthesis.

The genome of *T. cruzi* Tulahuen, as well as CL Brener, has a hybrid nature, in that it is comprised of two distantly related *T. cruzi* lineages [18]. We found higher rates of nucleotide substitutions for *DHOD1* versus *DHOD2* or *DHOD1* versus *DHOD3* than for *DHOD2* versus *DHOD3*, suggesting that *DHOD1* and *DHOD2* differ in evolutionary origin, whereas *DHOD3* was derived by gene duplication of *DHOD2*. These findings disagree with those obtained by comparing the three *ACT* gene loci. That is, the insertion of three nucleotides, encoding Ala265, was observed in *ACT2* and *ACT3*, while these two genes shared less similarity [9]. It is possible that *ACT3*and *DHOD3* were derived from partial duplication of the *pyr* gene cluster, including *ACT2* and *DHOD2*, but *ACT3* acquired more nucleotide substitutions than *DHOD3*. Further analyses are necessary to clarify the mechanisms of duplication and evolution of the *pyr* genes in *T. cruzi*.

Although expression profiles of the three DHODs should be examined in relation to parasite physiology, it is very difficult. We could not identify each *DHOD* transcript by single-stranded DNA conformation polymorphism (SSCP), nor could we isolate each of the DHOD isoforms, due to their very similar molecular weight and pI, from parasite extracts. Likewise, recent proteomic analyses in *T. cruzi* CL Brener failed to demonstrate presence of DHODs and their peptides in any developmental stages [19]. It is noteworthy that all three *ACTs* in *T. cruzi* Tulahuen are transcribed in all developmental stages at steady-state level [9]. Evidence of polycistronic transcription in *T. cruzi* makes it likely that *DHOD* and *ACT* genes are transcribed in the similar manner.

Despite the extensive nucleotide substitutions, the amino acids constituting the active site are completely conserved in all three *T. cruzi* DHODs. However, this does not necessarily mean that the enzymatic properties of the three DHODs are identical or very similar. We therefore determined the kinetic properties of the recombinant enzymes, all of which were expressed as flavoproteins and displayed fumarate-dependent DHOD activity, characteristic of family 1A DHOD. Interestingly, all three DHODs had a higher V_{max} at 37 °C than at 25 °C. Amastigotes rely on de novo pyrimidine synthesis, suggesting a requirement for accelerated de novo biosynthetic activity [14]. Thus, the preference of *T. cruzi* DHOD for 37 °C