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ORIGINAL ARTICLE

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Austronesian origin of the 27-bp deletion of the erythrocyte band 3 gene in East Sepik, Papua New Guinea inferred from mtDNA analysis

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Abstract The 27-bp deletion in the erythrocyte band 3 gene (B3Δ27) constitutes a genetic basis for Southeast Asian and Melanesian ovalocytosis. The distribution of B3Δ27 has been interpreted to reflect malaria selection or dispersal of the recent expansion of Austronesian-speaking populations. To explore these two hypotheses, we examined eight malarious populations of the East Sepik Province of Papua New Guinea (PNG) that speak both the Austronesian and Papuan languages. The B3Δ27 allele frequencies within populations were not positively correlated with malaria endemicities. In contrast, statistically significant geographical variations in the B3Δ27 allele distribution were observed. B3Δ27 was high (0.06–0.07) in the islands, intermediate (0.02–0.03) in coastal regions, but was absent or rare (0.00–0.01) in inland populations. Furthermore, the prevalence of the mitochondrial DNA region V 9-bp deletion, associated with the Austronesian expansion, was significantly correlated with that of B3Δ27. These results suggest that B3Δ27 was introduced by Austronesian-speaking people within the past 3,500 years and susequently expanded to populations along the coasts and islands of PNG. This study highlights the contribution of population origins, patterns of gene flow, disease selection and genetic drift in determining the genetic compositions of present populations.

Keywords Southeast Asian and Melanesian ovalocytosis · Malaria · Austronesian · Papua New Guinea · mtDNA

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Introduction

Natural selection by malaria has been generally recognized as the most important contributor to the evolution and maintenance of several polymorphic red blood cell abnormalities. Southeast Asian and Melanesian ovalocytosis (SAO) is one such red blood cell cytoskeleton abnormality characterized by oval-shaped erythrocytes (Lie-Injo 1976; Amato and Booth 1977). Early studies based on morphological criteria suggested that SAO traits confer protection against malaria parasite infection (Baer et al. 1976; Cattani et al. 1987) or in vitro *Plasmodium falciparum* merozoite invasion (Kidson et al. 1981).

After Jarolim et al. (1991) identified the molecular basis for SAO as a 27-bp deletion in the erythrocyte band 3 gene (B3Δ27), studies of morphologically defined SAO have been reassessed using this genetic criterion. No homozygous B3Δ27 genotypes have been observed, indicating lethality in the homozygous state (Liu et al. 1994). Although individuals heterozygous for B3Δ27 were not protected from uncomplicated malaria or high-density infection (Allen et al. 1999; Kimura et al. 2002), significant protection from cerebral malaria has been demonstrated (Genton et al. 1995; Allen et al. 1999). More recently, an in vitro study (Cortes et al. 2004) revealed that four out of five different *P. falciparum* parasite lines tested were not able to effectively invade B3Δ27 red blood cells. These results suggest that B3Δ27 has a selective advantage against at least some aspects of *P. falciparum* malaria, and that B3Δ27 can be maintained in malarious environments through balancing

selection, although it remains unclear what roles, if any, other *Plasmodium* species play in maintaining the B3Δ27 allele.

The distribution of the B3Δ27 allele expanding from the Philippines, Malaysia and Indonesia to Papua New Guinea (PNG) in the East and to Mauritius, South Africa, and Madagascar in the West (Jarolim et al. 1991; Ravindranath et al. 1994; Mgone et al. 1996; Kimura et al. 1998, 2003; Rabe et al. 2002) is coincident with the diasporas of Austronesian-speaking populations. In the Pacific, two waves of human settlement have been proposed, supported by evidence from archeological and linguistic studies. Humans first settled in New Guinea by 40,000 years before present (ybp; Groube et al. 1986), in the major islands of the Bismarck Archipelago by 35,000 ybp (Pavlides and Gosden 1994), and in the northern Solomon Islands by 32,000 ybp (Wickler and Spriggs 1988). This period of Pleistocene settlement is coincident with the current limit for Papuan languages. In contrast, Austronesian-speaking populations entered the Bismarck Archipelago from Southeast Asia around 3,500 byp, where they interacted with long-term residents and consequently settled in the islands beyond the Solomon Islands (Bellwood 1989). The expansion of the Austronesian people is correlated with the present distribution of specific mitochondrial DNA (mtDNA) lineages. In particular, the mtDNA region V 9-bp deletion has been found in Austronesian-speaking populations in north coastal and island PNG, Polynesia and Micronesia, but not in the Papuan-speaking New Guinea highlands (Stoneking and Wison 1989; Hertzberg et al. 1989; Lum and Cann 1998). Because of these distributions, the 9-bp deletion has been interpreted as a marker for the expansion of the Austronesian-speaking people into the Pacific (Merriwether et al. 1999).

Previous studies in PNG (Mgone et al. 1996; Kimura et al. 2003) revealed that the B3Δ27 allele frequency correlated with altitude, which is often inversely correlated with malaria endemicity, and was substantial (0.01–0.17) in all Austronesian-speaking populations. On the other hand, the B3Δ27 allele was absent or low (<0.01) in five out of six Papuan-speaking populations. The one notable exception was observed in Balimo, Western Province, where there was a relatively high (0.06) B3Δ27 allele frequency. These patterns in the distribution of B3Δ27 have led to two hypotheses: (1) positive selection resulting from malaria resistance (Mgone et al. 1996) and (2) dispersal of Austronesian-speaking peoples within the last 3,500 years (Kimura et al. 2003). These two hypotheses are difficult to distinguish, because Austronesian-speaking populations are restricted to the malarious islands and lowlands of PNG and are not found in the nonmalarious highlands. In addition, since there has been extensive gene flow between Austronesian and Papuan populations in islands and coasts of PNG (Lum and Cann 1998; Merriwether et al. 1999), analysis using Austronesian genetic markers to assess the Austronesian influence on these populations is desirable. To evaluate the validity of these hypotheses, we selected both Austronesian-speaking and Papuan-speaking populations along a fine scale transect within the malaria-endemic East Sepik Province of PNG, and determined the microgeographical distributions of the B3Δ27 allele, malaria endemicity, and the mtDNA 9-bp deletion, a genetic marker of the Austronesian influence.

Materials and methods

Field study

We conducted malariometric surveys of eight villages in the Wewak and Yangoru-Saussia Districts of East Sepik Province, PNG between August 2001 and February 2003. These eight villages extend along a transect from latitude 2°14′ to 4°5′ S, spanning (1) offshore islands of the Bismarck Sea ($n=2$, Walis and St Martins), (2) coastal lowlands ($n=2$, Dagua and Boiken), (3) the Prince Alexander foothills ($n=2$, Kaboibus and Jawia), and (4) the Sepik River Plain ($n=2$, Witupe and Kiniambu) ecological zones (Fig. 1). Four language groups are spoken in the study area: three Papuan languages (Boiken, Arapesh, and Abelam) and one Austronesian language (Kairiru).

To assess malaria endemicity, we determined parasite and spleen rates in children aged 2–9 years old (Snow

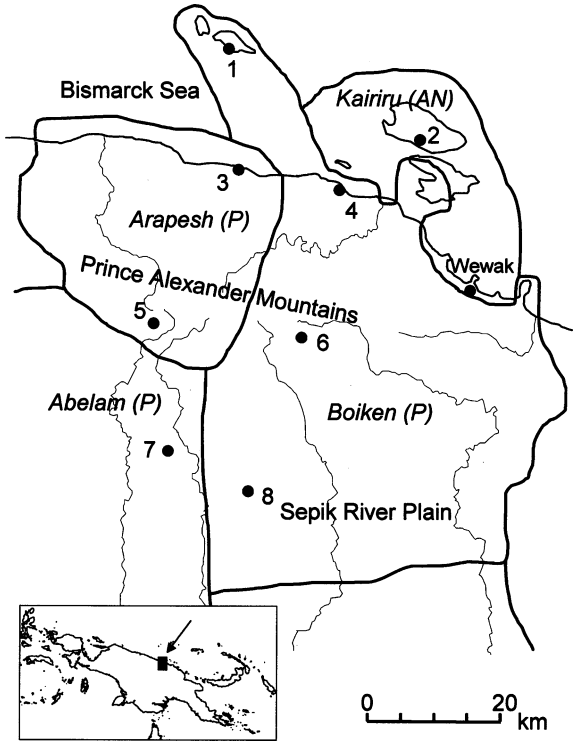

Fig. 1 Map showing the populations examined and the boundaries of language groups. The populations are numbered in ascending order of latitude south: *1* = Walis, *2* = St Martins, *3* = Dagua, *4* = Boiken, *5* = Kaboibus, *6* = Jawia, *7* = Witupe, *8* = Kiniambu. *Solid lines* show language group boundaries. *Italic letters* represent language groups: *AN* = Austronesian; *P* = Papuan

and Gilles 2002). We defined the parasite rate as the percentage of malaria-positive slides and the spleen rate as the percentage of individuals with palpable spleen. Blood samples were collected from the examinees with informed consent from their parents via study summaries translated into Niugini Pidgin and explained by FWH, IH, or local assistants. Thick and thin blood films were stained with 10% Giemsa and examined under a standard light microscope to determine parasitemia. We used Hackett's method to examine spleen enlargement in a recumbent position. Children detected with parasites were treated with antimalarial drugs according to the national PNG recommendations. Approval for the study was obtained from the National Department of Health Medical Research Advisory Committee of PNG and the Tokyo Women's Medical University Ethical Committee.

Genotyping of B3Δ27 and region V 9-bp deletion

One hundred children between 5 and 14 years old were selected with an unbiased sex ratio from each population. With informed consent, finger-prick blood samples (75–100 μL) were drawn into heparinized capillary tubes (Drummond Scientific Company, Broomall, PA, USA) and then transferred into plastic tubes containing dehydrated EDTA (BD microtainer 365973, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The tubes were kept at −20 °C prior to analyses. DNA was extracted from each blood sample using the chaotropes guanidinium and thiocyanate with size-fractionated silica particles (Boom et al. 1990). The 27-bp deletion in the erythrocyte band 3 gene and the 9-bp deletion of the mtDNA region V were assayed as described in Jarolim et al. (1991) and Hertzberg et al. (1989), respectively.

Statistical analysis

Pairwise relationships between the frequencies of the B3Δ27 allele, mtDNA 9-bp deletion, malaria endemicities (parasite and spleen rates), and the latitude south of each village were evaluated via Spearman's rank order correlation using SPSS 13.0J statistical analysis software (SPSS, Tokyo, Japan).

Results

Parasite rate in children aged 2–9 years ranged from 24 to 53% (Table 1). *P. falciparum* was the predominant species, accounting for 66% of infections, followed by *Plasmodium vivax* (29%) and *Plasmodium malariae* (5%). High spleen rates (55–90%) were also observed within all studied populations (Table 1).

Thirty-seven children (4.6%) of the 800 surveyed had the B3Δ27 allele. Consistent with previous studies, all of the individuals with the B3Δ27 allele were heterozygotes. Marked geographical variation in the B3Δ27 allele distribution was observed. Island villages, including the Austronesian-speaking population, had the highest B3Δ27 allele frequencies (0.06–0.07). In addition, the B3Δ27 allele was intermediate (0.02–0.03) in the Papuan-speaking coastal populations. In contrast, the B3Δ27 allele was absent or rare (0.01) in the more remote Papuan-speaking populations living in inland areas 20–50 km from the coast (Table 1). The B3Δ27 allele frequencies and latitude south were significantly and inversely correlated ($r = -0.805$, $p = 0.016$).

Although the mtDNA region V 9-bp deletion was found in all of the populations, like the B3Δ27 allele, its frequency was highest on the islands and coasts (0.13–0.25) and lowest in inland populations (0.02–0.08), with the highest frequency observed in St Martins, the Austronesian-speaking population (0.25; Table 1). A negative but not significant correlation between 9-bp deletion and latitude south was found ($r = -0.647$, $p = 0.083$).

The B3Δ27 and 9-bp deletion allele frequencies among the study populations were significantly correlated ($r = 0.884$, $p = 0.004$; Fig. 2). Negative correlations between the B3Δ27 allele frequencies and the two measures of malaria endemicity were observed: parasite rate; $r = -0.756$, $p = 0.030$ and spleen rates; $r = -0.342$, $p = 0.408$.

Discussion

In our study areas, statistically significant geographical variations were in the B3Δ27 allele distribution was observed. B3Δ27 was high (0.06–0.07) in the islands,

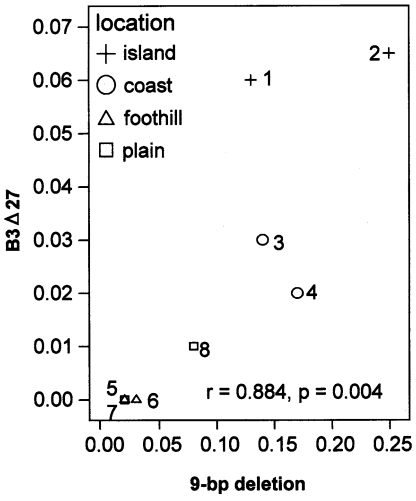

Fig. 2 Correlation between 27-bp deletion of band 3 gene (B3Δ27) allele frequency and mtDNA region V 9-bp deletion frequency. The populations are numbered in ascending order of latitude south: *1* = Walis, *2* = St Martins, *3* = Dagua, *4* = Boiken, *5* = Kaboibus, *6* = Jawia, *7* = Witupe, *8* = Kiniambu (see Fig. 1 for the study site location)

intermediate (0.02–0.03) in coastal regions, but absent or rare (0.00–0.01) in inland populations, suggesting a gradient in the allele frequency from the islands to the Sepik plain. Furthermore, a significant association between the B3Δ27 allele and 9-bp deletion frequencies was observed. These results support the hypothesis that these two alleles arrived in the Pacific with Austronesian-speakers, dispersed among the islands and coasts, but did not move inland due to limited gene flow. Previously studied populations along the north coast of PNG were characterized by ~40% 9-bp deletion frequencies (Stoneking and Wison 1989), approximately twice those of our island and coastal villages. The relatively low frequencies of 9-bp deletion we observed suggest that the populations we studied have received relatively large amounts of cumulative gene flow from inland, Papuan-speaking populations. The result is consistent with the historical and linguistic data on the expansion of Boiken speakers (Roscoe 1989). Interestingly, the oral tradition of Walis, the Papuan-speaking island population with the second highest frequency of the B3Δ27 allele (0.06) and a high frequency of the 9-bp deletion (0.13) recalls an intrusive movement of Boiken-speaking males (Anurim and Kabaru 1980). This account may reflect the local replacement of an Austronesian language by the currently spoken Papuan language. Furthermore, the Papuan-speaking population in Balimo, which has a relatively high (0.06) B3Δ27 allele frequency (Mgone et al. 1996), cultivates the ritual sedative plant kava (*Piper methysticum*) that was thought to have been domesticated by Austronesian speakers in Vanuatu (Lebot et al. 1997). Thus, horticultural evidence indicates that in the past it was a region of cultural and likely genetic exchange with Austronesian-speakers.

The distribution of the 9-bp deletion we observed is consistent with the dispersal and gene flow of Austronesian speakers; however, the results may also reflect stochastic processes inherent in the limited number of populations and genetic loci examined. By examining higher resolution mtDNA data as well as paternally inherited Y-chromosomal haplotypes, we expect to attain a deeper understanding of gene flow within the East Sepik Province.

The high spleen rates observed ($>50\%$) indicate a recent history of intense, hyperendemic malaria infections in all studied populations. Spleen rates provide a preferable way to assess malaria endemicity because parasite rates at a given location often vary significantly over time. Because of the expected loss of B3Δ27 alleles through homozygous lethality, without positive selection the allele should decline to <0.02 within 45 generations (or about 1,400 years) regardless of the initial frequency. Substantial (0.02–0.07) B3Δ27 allele frequencies in the island and coastal populations suggest that the B3Δ27 allele has been maintained by malaria selection. However, this malaria selection hypothesis does not explain why the B3Δ27 allele was absent or almost absent in inland populations, despite the fact that these areas have the highest malaria endemicities as measured via both parasite and spleen rates (Table 1).

Theoretically, the expected strength of genetic drift is inversely proportional to population size. Therefore, if stochastic loss due to drift is responsible for the absence of the B3Δ27 allele inland, but not on the coasts or islands, then inland populations must have been relatively small and vulnerable. In fact, the inland populations in Prince Alexander Range foothills have been historically large and dense (Allen 1983). Therefore, genetic drift is not likely to be the major cause of the absence of B3Δ27 in the inland populations. Alternatively, if the current distribution reflects the dispersal of coastal people, then drift acting on the small numbers of individuals migrating from the coast may have hindered the establishment of the B3Δ27 allele inland.

If the B3Δ27 allele was introduced to PNG by Austronesian-speaking people within the past 3,500 years and expanded into descendant populations as our analyses suggest, it should also be present in malarious Vanuatu, first settled by Austronesian-speaking people ~3,000 ybp (Summerhayes 2001). Further assays of the B3Δ27 allele in Vanuatu populations that are currently underway will examine the prediction.

Table 1 Twenty-seven base pair deletion of the erythrocyte band 3 gene (B3Δ27), mtDNA region V 9-bp deletion, and malaria endemicities in the study populations

Population[a]	B3Δ27		9-bp Deletion		Malaria-positive in 2–9 years		Spleen enlarged in 2–9 years	
	n/Total	Allele frequency	*n*/Total	Frequency	*n*/Total	%	*n*/Total	%
Walis	12/100	0.060	13/100	0.130	42/176	23.9	105/169	62.1
St Martins	13/100	0.065	25/100	0.250	117/384	30.5	251/372	67.5
Dagua	6/100	0.030	14/100	0.140	182/605	30.1	356/533	66.8
Boiken	4/100	0.020	17/100	0.170	124/349	35.5	290/346	83.8
Kaboibus	0/100	0.000	2/100	0.020	108/246	43.9	208/232	89.7
Jawia	0/100	0.000	3/100	0.030	169/528	32.0	311/499	62.3
Witupe	0/100	0.000	2/100	0.020	168/318	52.8	253/284	89.1
Kiniambu	2/100	0.010	8/100	0.080	41/124	33.1	64/116	55.2
Total	37/800	0.023	84/800	0.105	951/2,730	34.8	1,838/2,551	72.1

[a]The populations are arranged in ascending order of latitude south

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