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A PRELIMINARY STUDY ON THE DISTRIBUTION OF BETA DEFENSINS COPY NUMBER VARIABLE GENE IN DIFFERENT ETHNICS OF SARAWAK, MALAYSIAN BORNEO

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Abstract: The indigenous groups of Sarawak that are well-known for diverse ethnicity featuring different cultures, languages and lifestyles, are among the earliest resident of Malaysian Borneo. The high survivorship of these early inhabitants had triggered an interest on their ability to develop immune or defense mechanisms towards the environment. Beta defensin is one of the well-studied adaptive immune functional gene of human. Beta defensins gene family plays a major role in innate and adaptive immune systems that are located in a cluster of at least seven genes on chromosome 8p23.1 with highly variable copy number. The ability of the indigenous people populating new settlements has been linked to their resistance or susceptibility towards certain disease which is influenced by the effectiveness of beta defensins adaptability. Hence, investigation on the distribution of beta defensins copy number variable gene in ethnics of Sarawak was conducted by investigating two microsatellite regions within the gene cluster. The result from 78 respondents comprising of the Iban, Bidayuh, Selako, Bugis and Jawa on the immediate copy number typing range from 2 to 8 copies. Interestingly, the copy numbers are not unique to the ethnics. They show a broad overview of shared copy number typing between them. All the sampled ethnics showed an agreement of copy number typing with modal copy of four based on EPEV1 and EPEV3 except for the Selako population. Pearson's correlation shows excellent agreement between the samples with R^2=1. The differences in copy number typing between populations were mostly due to genetic drift within the population. Natural selection had caused small populations to develop defence mechanisms and adaptability towards the environment. Furthermore, intercultural marriage within small populations has discouraged gene flow and limits genetic drift which later becomes a mechanism of evolution for beta defensins copy number gene. This study highlights the diversity and distribution of beta defensins copy number variable gene between ethnics and localities. These may serve as the basis for our understanding of the evolution of the beta defensins gene within ethnicities and their ability in expansion of population due to resistant towards the environmental stress and natural selection.

Keywords: Indigenous people, beta defensins, immune defence, copy number, microsatellites assay, environment.

Introduction

Sarawak has a diverse ethnic population that comprising of over 40 indigenous groups. Each ethnic has their own distinct language, culture and lifestyle. This makes the demography distinct and unique. The biggest native group in Sarawak is the Iban community, which also known locally as the Sea Dayak (Jawan, 2006). Their social structures were organised around the efficient cultivation of hill rice, which was a crucial staple food for the community's survival in the past. The Iban social hierarchy can be divided into three levels: the nuclear family, the longhouse community and the territorial tribe (Kedit, 2006). The Bidayuh community consists of about 8% of the population in Sarawak. They are mostly concentrated on the west end of

Borneo and occupies the hill counties such as Lundu. The Selako community feature different descent and migration histories as observed in the verbal tradition and customs (Demographics of Sarawak, 2017). However, typically they are neighbours to Bidayuh's as they occupy almost the same living and cultivating area. Thus, they are locally known as the Land Dayak together with the Bidayuh (Boulanger, 2009; Vasudevan *et al*., 2011). As for the Jawa, majority of the community originated from the Central Java. Most of them migrated to Sarawak in the early days as indentured servants who worked in the rubber plantations (Alexander, 2006). The Bugis community originated from the southwestern province of Sulawesi, renowned as the adventurous seafarer and traders in the Malay Archipelago. In Sarawak, Bugis community can be found along the coast line (Peletz, 2009).

Most of the ethnics were said to have migrated to Sarawak from neighbouring islands surrounding Borneo, causing differences in their physical features and lifestyles (Wright *et al*., 1971). The organisational structures among ethnics are based on spatial divisions and social inter-relationships among ethnics. The ability of the ethnic groups to tolerate the surrounding and become local inhabitants are believed to be due to natural selection during then. The selection pressure had triggered the immune defence mechanism within their body to adjust and suit to its surroundings. Furthermore, lack of gene flow and limited genetic drift are speculated to be the reason for evolution of the defensins gene in early inhabitants of Sarawak, Malaysian Borneo.

Defensins are grouped into the family of peptides that play an important role in the innate immune response which have a broad spectrum of antimicrobial activities against bacteria and fungi (Del Pero *et al*., 2002). Three families of mammalian defensins have been identified including alpha-defensins, beta-defensins and theta-defensins, presumably derived from a common ancestral defensins (Das *et al*., 2010). The ability of defensins in becoming the antimicrobial agents to most gram-negative and gram-positive bacteria as well as other fungi and viruses makes it beneficial to study the construction of the defensins genes. Information obtained from the UCSC genome browser shows the entire genes (alpha and beta defensins chromosome 8p23.1) on *Homo sapiens sapiens* which spanned about 2 Mb (Kent *et al*., 2002).

Beta defensins contribute to mucosal and epithelial defence, also acting as signal molecules for cellular components of innate and adaptive immunity (Crovella *et al*., 2005). Beta defensins play a major role in innate and adaptive immune systems through chemoattraction of dendritic cells, memory T cells (Yang *et al*., 1999), and human neutrophils (Niyonsaba *et al*., 2004). Disruption of beta defensins genes in human has implication in various human diseases (Radhakrishnan *et al*., 2005) which makes it essential to evaluate the functional ability of this gene in human.

Human beta defensins are found on chromosome 8p23.1 (Ganz *et al*., 2003), with a diploid copy number commonly ranging between 2 and 7 copies (Hollox *et al.,* 2003), contrasting with the beta defensin cluster on chromosome 20 which are not variable in gene copy number. The beta defensins genes on 8p23.1 include *DEFB4, SPAG11, DEFB103, DEFB104, DEFB105, DEFB106* and *DEFB107*, with the exception of *DEFB1* are on a large repeat unit that is variable in copy number.

Microsatellite analysis is well-known as short tandem repeats (STRs). They are usually of repetitive DNA motif ranging with about 2 to 5 bp. The repeats can sometimes occur up to 50 times (Turnpenny & Ellard, 2005). Microsatellite alleles are highly informative and unique between individuals.

Therefore, this study is to investigate selected ethnics in Sarawak in order to characterise the beta defensin copy number variable diversity among ethnics using microsatellite markers. Characterisation of the beta defensins copy number variable gene among ethnics may provide answers to the ability of these ethnics to survive the environment.

Methodology

Sample Collection

Ethical approval and permission were obtained from the respective organisations; Universiti Malaysia Sarawak and Sarawak Health Department (UNIMAS/TNC(AA)-03.02/06-11 Jld. 2(93)) to collect human samples from different ethnics of Sarawak. Each respondent had to be interviewed to make sure (the pure and unmixed respondent) their parents and farther common ancestors come from the same ethnic. Mixed respondents were excluded from this study. Written informed consent form were obtained from each subject before commencement of the study. A pre-questionnaire was prepared in Bahasa Melayu and English to collect history of medical status, lifestyles and other demographic data. Blood samples (1 – 3 ml) were taken by a qualified medical practitioner in the research team and were treated and preserved in BD Vacutainer® K2 EDTA tubes.

DNA Isolation

The DNA from blood sample was extracted and purified by using the QIAmp DNA Mini Kit (QIAGEN, Germany). The method of the extraction followed the manufacturer protocol.

Copy Number Typing Using Microsatellite Assay

Two microsatellite primer sets (Table 1), EPEV1 (Hollox *et al*., 2003) and EPEV3 (Abu Bakar *et al*., 2009) that were fluorescently-labelled with HEX marker were used to perform capillary electrophoresis. The 10X PCR mix were 5 µl 2X Prime Taq premix, 0.5 µl 10 uM EPEV labelled forward primer and EPEV reverse primer, 2.0 µl ultrapure water and 2.0 µl 10 ng DNA template. The PCR amplification profile were denaturation at 95 ºC for 1 min, annealing at 58 ºC for 1 min, extension at 72 ºC for 1 min; repeated for 25 cycles and final extension at 72 ºC for 20 mins.

Data Analysis

The amplified microsatellite products were resolved by capillary electrophoresis. Peaks were visualised using Peak Scanner™ version 1.0 to collect the product size and peak height. The peak height ratios of all alleles to the smallest allele were calculated to infer the copy number. The total allelic ratio calculated is considered as integer copy number. The allelic size were also collected to determine the inheritance pattern among the ethnics. The electropherogram in Figure 1 showed example of the amplified peak from a sample. Ratio of each true peak to the smallest peak (1:1) in EPEV3 resulted in a total copy number of 2. The adjacent peak to the true peak is considered as the stutter peak. Stutter peaks are slippage from the original peak and will be added back to the true peak for correct ratio calculation. However, with reference to EPEV1, 6 copies were seen from the calculated ratio (2:1:3). Microsatellite analysis allows every allele to be multiplied with integer copy with reference to the inferred ratio from both markers. Thus, the sample from Figure 1 is assumed to have 6 copies.

Table 1: Primer sequence for EPEV1 and EPEV3

Primer	Primer sequence (5'- 3')	Expected product size
EPEV1	Labelled forward:	
	GGCAGTATTCCAGGATACGG	
	Reverse:	160 to 193 bp
	GAACAATTAGATATCCCTATGC	
EPEV3	Labelled forward:	
	GATACTGTGAACTACAGATCAC	
	Reverse:	125 to 151 bp
	CTGCCCTGATTCAGTATTGAAC	

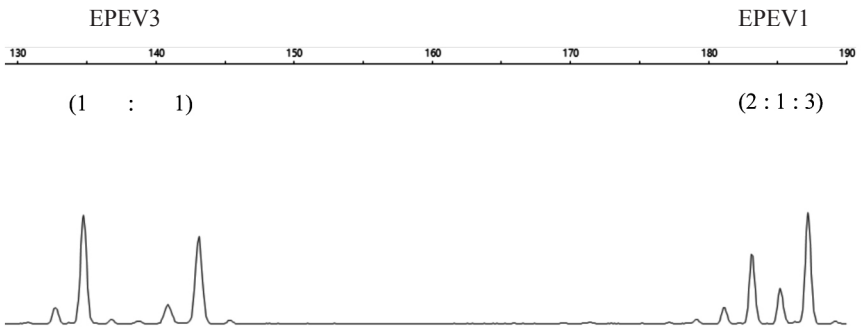

Figure 1: Electropherogram as recorded by Peak Scanner™ for 6-copy sample typed for EPEV1 and EPEV3. Allele in EPEV3 was referred as 1 allele has three copies (2 x 3 ~ 6)

Results

Respondents were grouped into five ethnics; Iban, Bidayuh, Selako, Jawa and Bugis. All ethnics except the Iban comprises of 13 samples. However, the Iban comprised of two localities; Rembus and Sebayor, with equal number of respondents, making a total of 26 Iban samples. All 78 samples shows successful copy number typing for both EPEV1 and EPEV3. Allelic size and ratio between EPEV1 and EPEV3 is as shown in Table 2. Only unique allele size is shown for respective copy numbers as few of the samples have similar allele size and ratio.

Table 2: Allelic size and allelic ratio for EPEV1 and EPEV3 inclusive of the inferred copy number (CN)

Number of respondents Iban (Rembus)	Inferred CN	EPEV1 Allele size (Ratio)	EPEV3 Allele size (Ratio)
1	3	171: 183 (2:1)	141: 143: 145 (1:1:1)
8	4	171: 181: 185 (1:2:1)	141: 143: 145 (2:1:1)
		183: 185: 189 (2:1:1)	137: 139: 141: 145 (1:1:1:1)
		171: 189: 191 (1:1:2)	141: 143: 147 (1:2:1)
3	5	171: 183: 185: 191 (1:1:2:1)	137: 141: 145 (1:2:2)
		171: 187: 189 (2:2:1)	135: 141: 143: 145 (2:1:1:1)
		171: 183: 185: 189 (1:1:2:1)	137: 141: 145 (1:2:2)
1	6	171: 185: 187 (2:1:3)	137: 139: 141: 143 (3:1:1:1)
Iban (Sebayor)			
3	3	171: 183 (2:1)	141: 143 (2:1)
		183: 187: 189 (1:1:1)	141: 145 (1:2)
		185: 187 (2:1)	141: 145 (2:1)
6	4	183: 185: 187: 191 (1:1:1:1)	135: 139: 141: 145 (1:1:1:1)
		185: 187: 191 (2:1:1)	143: 145 (1:3)
		185: 187: 189 (2:1:1)	139: 143: 145 (2:1:1)
		171: 185: 187 (1:2:1)	135: 139: 143: 145 (1:1:1:1)
		183: 185: 189 (1:2:1)	141: 145 (3:1)

		171: 185 (3:1)	141: 145 (3:1)
1	5	171: 185: 187: 191 (2:1:1:1)	141: 143: 145 (2:1:2)
2	6	171: 183: 185: 189 (1:3:1:1)	139: 143: 145 (2:1:3)
		171: 183: 185 (2:3:1)	141: 143 (2:1)
1	7	171: 183: 185: 187 (2:1:3:1)	141: 143: 145 (3:1:3)
Bidayuh (Mongkos)			
1	2	171: 181 (1:1)	141: 143 (1:1)
2	3	171: 179: 183 (1:1:1)	141: 143: 145 (1:1:1)
		171: 183: 187 (1:1:1)	139: 141: 143 (1:1:1)
7	4	183: 185: 189 (2:1:1)	137: 141: 143 (1:2:1)
		171: 183: 187 (1:1:2)	135: 137: 139: 143 (1:1:1:1)
		171: 183: 185 (1:2:1)	139: 141: 143 (1:2:1)
		171: 183: 187 (1:2:1)	139: 143: 145 (1:1:2)
		171: 185: 189 (1:1:2)	135: 139: 141 (1:1:2)
1	5	185: 189 (1:4)	135: 141: 143: 145 (2:1:1:1)
2	6	171: 183: 185 (1:4:1)	135: 141: 143 (2:1:3)
		171: 183: 185 (1:3:2)	135:143: 145 (1:3:2)
Selako (Pueh)			
7	3	171: 183: 185 (1:1:1)	141: 143 (1:2)
		171: 185: 187 (1:1:1)	141: 145 (1:2)
		171: 187 (1:2)	141: 143: 145 (1:1:1)
3	4	171: 185: 187: 189 (1:1:1:1)	135: 139: 143: 145 (1:1:1:1)
		171: 185: 187 (1:1:2)	139: 141: 143 (2:1:1)
		171: 183: 185 (1:2:1)	141: 143 (1:1)
2	5	171: 183: 185 (1:3:1)	141: 143: 145 (2:1:2)
		171: 183: 185 (1:2:2)	139: 141: 143 (2:2:1)
1	6	171: 185: 187 (2:1:3)	135: 141: 143: 145 (1:1:2:2)
Jawa (Batu 10)			
2	3	171: 187: 189 (1:1:1)	141: 143: 145 (1:1:1)
		185: 187: 191 (1:1:1)	139: 141: 143 (1:1:1)
6	4	171: 183: 185: 191 (1:1:1:1)	135: 137: 139: 143 (1:1:1:1)
		185: 187: 189 (1:2:1)	135: 141: 145 (2:1:1)
		171: 183: 185 (1:1:2)	139: 143: 145 (2:1:1)
		183: 185: 189 (1:2:1)	139: 141: 143 (2:1:1)
		171: 185: 187: 191 (1:1:1:1)	143: 145 (2:2)
		171:185:187 (2:1:1)	141: 143: 145 (2:1:1)
2	5	171: 183: 185 (1:3:1)	135: 139: 141: 143 (1:2:1:1)

		171: 183: 185 (2:2:1)	141: 143: 145 (1:2:2)
3	6	171: 183: 185 (1:3:2)	135: 139: 141: 143 (2:1:1:2)
		171: 183: 185: 187 (1:3:1:1)	141: 143: 145 (2:2:2)
		171: 183: 185: 187 (1:2:2:1)	143:145 (1:2)
Bugis (Sadong Jaya)			
3	2	171: 191 (1:1)	141: 143 (1:1)
2	3	171: 189: 191 (1:1:1)	141: 143: 147 (1:1:1)
		171: 185: 189 (1:1:1)	141: 143 (1:2)
5	4	185: 187: 189 (1:2:1)	139: 141: 143 (2:1:1)
		171: 183: 185 (1:1:2)	139: 141: 143 (2:1:1)
2	6	171: 183: 185 (1:4:1)	141: 143: 147 (1:4:1)
		171: 189: 191 (1:1:1)	139: 143: 145 (2:1:3)
1	8	171: 177: 179: 183 (1:4:2:1)	139: 141: 143 (6:1:1)

* Number of respondents refer to total number of respondents with the same copy number. However, samples of repeated allele size and ratio are not stated, with only the unique allele size and ratio shown.

Allele size 171 is well distributed in samples typed with EPEV1 while allele size 141 is commonly observed in EPEV3. A few samples showed one allele one copy pattern for both EPEV which is less informative for copy number typing due to similar ratio. However, copy numbers are determined based on both EPEV. For example, one Iban (Sebayor) sample gives a total ratio of 3 copies for EPEV3. Since EPEV1 show a true ratio of six, thus, the sample is inferred as six copies; with EPEV3 is assumed to have 2 copies for every allelic ratio (3 x 2 ~ 6).

Table 3 indicates the cross-tabulation of EPEV1 and EPEV3 for ethnics sampled. From the table, copy number 4 is the modal copy number, based on the results obtained using EPEV1 and EPEV3 where 4 is the most frequent copy number for the Sarawak ethnics sampled. However, there are few samples that show high copy number and represented by only a single sample for both 7 and 8 copies. Figure 2 shows the agreement of beta defensin copy number for the ethnics sampled in Sarawak. The data is presented based on Pearson's correlation. From the Figure 2, the agreement of both EPEV is excellent (R^2=1). According to Abu Bakar *et al.* (2008), the results from EPEV1 and EPEV3 should show the same copy number since both templates primer reside within the same locus of beta defensin.

Table 3: Cross-tabulation of EPEV1 and EPEV3 for Peak Height

		EPEV 1							
		2	3	4	5	6	7	8	Total
EPEV 3	2	4							4
	3		17						17
	4			35					35
	5				9				9
	6					11			11
	7						1		1
	8							1	1
	9								0
	10								0
Total		4	17	35	9	11	1	1	78

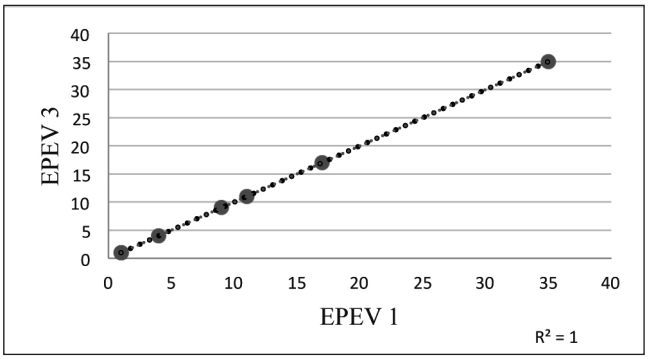

Figure 2: Agreement between EPEV1 and EPEV3 among ethnics

Table 4 showed the agreement of inferred copy number for EPEV1 and EPEV3 for the Sarawak ethnics sampled. As observed, Pearson's correlation results had shown maximum similarity (Figure 2). The Iban population from two localities within the same district shows a slight difference in the copy number count between the two localities as shown in Figure 3. Based on the data, each ethnic had different composition of copy number and most of the ethnics had copy number of 4 as their modal except for the Selako, whom had copy number of 3 as the modal copy number. Figure 4 shows that there is one sample having a copy number of 7 and 8 respectively. Samples having 8 copies is seldom found in Malaysian population since normal Malaysian copies can be found in range of 2 – 7 copies per individual. The Iban shows higher frequency due to samples were collected from two localities.

Table 4: Distribution of copy number among Sarawak ethnics

Marker	**Ethnic**	**Copy number**							**Total**
		2	3	4	5	6	7	8	
	Iban (Rembus)	0	1	8	3	1	0	0	13
	Iban (Sebayor)	0	3	6	1	2	1	0	13
	Bidayuh	1	2	7	1	2	0	0	13
EPEV 1	Selako	0	7	3	2	1	0	0	13
	Jawa	0	2	6	2	3	0	0	13
	Bugis	3	2	5	0	2	0	1	13
	Total	4	17	35	9	11	1	1	78
	Iban (Rembus)	0	1	8	3	1	0	0	13
	Iban (Sebayor)	0	3	6	1	2	1	0	13
	Bidayuh	1	2	7	1	2	0	0	13
EPEV 3	Selako	0	7	3	2	1	0	0	13
	Jawa	0	2	6	2	3	0	0	13
	Bugis	3	2	5	0	2	0	1	13
	Total	4	17	35	9	11	1	1	78

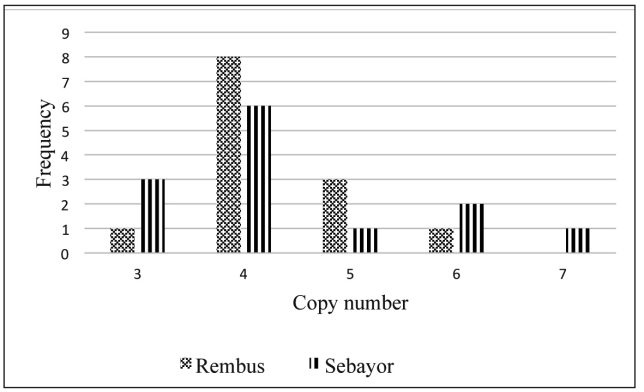

Figure 3: Distribution of copy number among the Iban from two localities; Sebayor and Rembus for EPEV1 and EPEV3

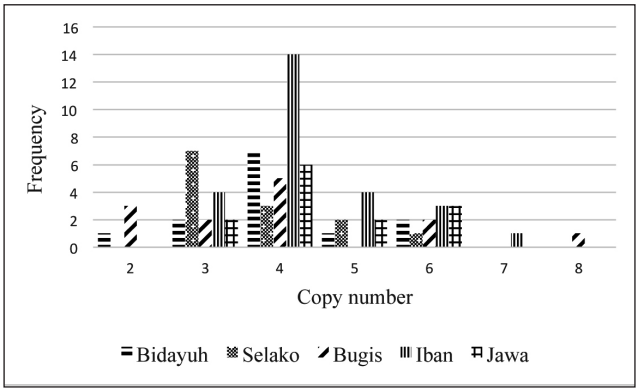

Figure 4: Distribution of copy number among five ethnics from six localities for EPEV1 and EPEV3

Discussion

Microsatellite analysis is proven to be able in deducing the copy number between ethnics. One of the two markers may act as reference towards the other. However, since microsatellite analysis has the ability to be factored to any integer, the use of other analysis method should be unified for validation. This is essential in documentation of the real copy number between ethnics without any false-positive.

Copy number typing using microsatellite assay agrees with previous studies reported by many authors with the modal copy number four (Hollox *et al.,* 2003; Abu Bakar *et al*., 2009). Copy numbers are usually normally distributed within a population with three, four and five copies commonly found in the evaluated samples (as shown in Figure 4). Modal copy are always within its range as copy number are significantly known to influence the prevalence of certain diseases in any given case of elevated or reduced copies (Bentley *et al*., 2009; Fellerman *et al*., 2006). However, reduced copy number in the Selako population sample from Pueh is an interesting finding that may be real or due to the effect of small sample size analysed (Hollox *et al*., 2008; Aldhous *et al*., 2010). This is since small sample size tend to be biased and should be interpreted with caution (NurWaliyuddin *et al*., 2015).

Since the Selako samples were collected from only a single longhouse, thus, the respondents are genetically related to each other with potentially very minimal gene flow given their closed social behavior. In the early days,

the Selako had migrated in small groups from one settlement to another, thus, this activity may have reduced the genetic variation within the population (Fix & Lie-njo, 1975). Therefore, variation in modal copy number detected could also be due to genetic drift in small populations (Nguyen *et al*., 2006). Different admixture level within the Selako are probably the contributing factor influencing genetic variation within the population. The limited gene flow had significantly changed genetic composition within the population (NurWaliyuddin *et al*., 2014). However, it does not answer whether the detected copy number originates from the ancestral gene pool or is due to variation from the ancestral gene pool. Nevertheless, this natural selection processes due to cultural or geographical isolations that occur in CNVs of certain organisms could potentially lead to greater evolutionary changes within the family (Perry, 2008).

Though some ethnics sampled are possibly of limited gene flow, the genetic variation recorded could still relate to the diversity in the copy number count among the ethnic or localities as observed in the Iban population. Pedigree studies have suggested a mutation rate of the human locus of around 0.7% per generation (Abu Bakar *et al*., 2009). Alleles of different copy number, while clearly inherited in a Mendelian manner, combined to give a diploid copy number genotype which can vary substantially between parent and child, and between siblings due to normal segregation of homologous chromosomes (Wain *et al*., 2009). This may explain the slight different in copy number within an ethnic.

Small sample size is the limiting factor in determining the cause of disagreement of copy number typing between EPEV1 and EPEV3 in the Jawa population. However, adjustment with Pearson's correlation had eliminated the constraint with the Jawa population with no significant difference. Though copy number typing can be used in measuring the genetic variation within populations, the results are sometimes not consistent and irreproducible (Bustamante *et al*., 2012; Hardwick *et al*., 2011; Hollox, 2010). The inconsistencies in copy number typing may be the reason for disagreement of the typed copy number within an ethnic based on the two markers.

This first documentation of beta defensins copy number within Sarawak ethnics has shown a range of 2 to 8 copies which is congruent with many previous studies. Although the copy number variable of beta defensins differ between studies, a significant copies of 2 to 12 diploid copies per genome are widely seen in most populations in the United Kingdom (Abu Bakar *et al*., 2009; Fode *et al*., 2011; Hardwick *et al*., 2011; Hollox, 2008). Hollox (2008) has documented the beta defensins distribution of seven populations recording a common copy number of 2 to 7 have been established in several HapMap populations. However, Chinese and Yorubans showed a slight increase between 3 and 7 as compared to the European populations.

Sarawak is located within the Asian region and thus, the current studied population can further be classified as the Asian population. Therefore, beta defensins copy number distribution within Asian population are less than the ones reported in the European population. The reason to differences in copy number variable beta defensins gene was speculated either to be due to genetic drift copies, differential selection pressure, differential rate of mutation, or an artifact of the small sample size (Hollox *et al*., 2008). These speculations were made due to level of gene expression strongly correlated with copy number variation (Hollox *et al.,* 2003). Yet, this remain to be further proven as the current study focuses only on limited samples and a small populations within Sarawak.

Conclusion

The present copy number typing using two microsatellite markers; EPEV1 and EPEV3 has been able to provide an insight on the diversity of copy number among ethnics in Sarawak. Beta defensin copy number variable gene obtained was a modal copy of four which is congruent with previous studies. Nonetheless, some

adjustments in copy number typing are observed within the samples. However, the reliability of copy number typing is still not fully resolved with only microsatellites analysis. Other approaches such as Paralogue Ratio Test (PRT) and Real Time PCR could aid in quantification of the real copy number in beta defensins gene. In addition, larger sample size from various localities should be incorporated in the future. A broad overview on the effect of genetic variation in beta defensins among ethnics may affect their ability in adapting towards environmental stress and natural selection. In all, this study had documented the existing beta defensin copy number variable gene within the ethnics of Sarawak.

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