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Microsatellite-based evidences of genetic bottlenecks in the cryptic species "*Andrographis paniculata* Nees": a potential anticancer agent

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Abstract *Andrographis paniculata* (AP) is a medicinal plant species introduced into Malaysia. To address the genetic structure and evolutionary connectedness of the Malaysian AP with the Indian AP, a DNA sequence analysis was conducted based on 24 microsatellite markers. Out of the 24 primer sets, seven novel microsatellite primers were designed and amplified intra-specifically according to the available Indian AP sequences at the National Centre for Biotechnology Information (NCBI), where 17 of them were amplified using the cross-species strategy by employing the primers belonging to *Acanthus ilicifolius* Linn (Acanthaceae) and *Lumnitzera racemosa* Wild (Combretaceae). The primers were then applied on the Malaysian AP accessions. Sixteen of the new microsatellite loci were amplified successfully. Analysis of these microsatellite sequences, revealed some significant differences between the Indian and Malaysian AP accessions in terms of the size and type of the repeat motifs. These findings depicted the cryptic feature of this species. Despite identifying several heterozygous alleles no polymorphism was observed in the detected loci of the selected accessions. This situation was in concordance with the presence of "fixed heterozygosity" phenomenon in the mentioned loci. Accordingly, this was fully consistent with the occurrence of the genetic bottleneck and founder effect within Malaysian AP population. Apart from the amplification of new microsatellites in this species, our observations could be in agreement with the risk of genetic depletion and consequently extinction of this precious herb in Malaysia. This issue should be taken into consideration in the future studies.

Keywords Genetic bottleneck · Founder effect · Microsatellite · Mutation · Cryptic species

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Introduction

Andrographis paniculata ($2n = 50$) known as the king of bitters in English, is a medicinal plant belonging to the family Acanthaceae. Even though the herb is famous due to its pharmaceutical effects, the agricultural and industrial applications have been taken into consideration recently [1]. Historical background of the relations between India and Malaysia supports the idea that AP was imported for medicinal purposes by the Indians who were brought from the southern region of India during the British colonization. These introduced genotypes were then distributed

throughout Malaysia along with the Indians settlement, and consequently, some new genotypes were generated through mutation and occasional crossing [2]. However, the genetic structure of these accessions may be stable without any gene flow into them [3]. On the other hand, agro-morphological and phytochemical characteristics of Malaysian APs are almost same as the Indian ones and this is in accordance with the idea describing India as the origin of Malaysian APs [4]. Microsatellite markers or SSRs (Simple Sequence Repeats) have proven to be greatly effective molecular tools due to their high reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage [5]. The DNA sequence analysis using microsatellite markers can reveal variations of the repeat motifs in comparison with the original lineages.

Study on the cryptic species (i.e. two or more distinct species that are very similar to each other to the extent that phenotypically are indistinguishable, whilst are speciously classified under one species name) has improved exponentially over the past two decades, by the increasing availability of DNA sequences [6]. Identifying and studying the cryptic species with complex evolutionary phases contribute to meeting conservation targets of biodiversity significantly. The introduced invasive species are one of the main reasons which cause population regressions among the native species in wild habitats. Misidentification of important species with cryptic complexes can lead to serious negative impacts on their conservation [7, 8]. Basically, investigation of the cryptic diversity has been concentrated in the temperate zones, whereas it remains undecided in the tropics, which are home to more than two-thirds of the world's defined diversity [8].

The present research was aimed to consider the occurrence of genetic bottleneck and its consequences on the Malaysian AP population. Concurrently, the genetic relationship between the Malaysian AP accessions and their Indian ancestors from Kerala was investigated. As it was declared previously, these accessions have been introduced to Malaysia most probably by the Indian emigrants from the southern parts of India (especially from the state of Kerala), where the first batch of AP's microsatellites have been reported from [9]. Therefore, the first part of this research was conducted using intra-specific microsatellite amplification strategy. Moreover, the genetic structure and the level of heterozygosity of the Malaysian AP accessions (as an unexplained case up to this point) were scrutinized using cross-species microsatellite amplification approach. The present upshots are the first evidence that may provide a fundamental knowledge of the evolutionary pathway and cryptic aspects of this unique plant species in Malaysia.

Materials and methods

Plant material and DNA extraction

Fresh leaves of seven AP accessions representing six states of Peninsular Malaysia, including accessions 11179 (Selangor), 11216 (Negeri Sembilan), 11261 (Perak), 11313 (Pahang), 11322 (Pahang), 11344 (Kelantan) and 11350 (Terengganu) were used individually for this study (Table 1). The modified Doyle and Doyle CTAB-based DNA extraction protocol was employed to extract the genomic DNA from AP's fresh leaves [10]. The purity of the entire extracted DNAs was assessed by calculating the OD260/OD280 ratio determined with the Nanodrop, Model ND1000 (Table 1).

PCR amplification and primer designing

The PCR reactions were performed in a total volume of 25 μL for each reaction using the Dream Taq® PCR kit (Fermentas GmbH, Germany). The PCR master mix optimization was carried out based on the manufacturer's instruction. The mixture contained Dream Taq® PCR Buffer (1×), 200 μM PCR nucleotide mix (dNTP), $MgCl_2$ (1.5 mM), primer (0.4 μM), 1.25 u of Taq DNA polymerase (Fermentas GmbH, Germany) and 50 ng of genomic DNA. A negative control (excluding template DNA) was performed in each set of reaction to detect contamination. The initial PCR optimization was carried out using gradient PCR amplification. Twelve annealing temperatures of between 52

Table 1 The quality and quantity of total genomic DNA of the seven Malaysian AP accessions

Code	Accession	State	OD 260	OD 280	Concentration (μg/mL)	260/280
1	11179	Selangor	0.302	0.173	1809	1.74
2	11216	Negeri Sembilan	0.657	0.344	1866	1.91
3	11261	Perak	0.308	0.163	1615	1.89
4	11313	Pahang	0.362	0.206	3280	1.76
5	11322	Pahang	0.895	0.451	4322	1.98
6	11344	Kelantan	0.666	0.342	3210	1.94
7	11350	Terengganu	0.630	0.331	3177	1.90

and 64 °C were utilized to complete the PCR program using a gradient Thermal Cycler (Biometra-Germany) with an initial step of pre-denaturation for 8 min at 94 °C, followed by 50 cycles of 10 s at 94 °C (Denaturation), 15 s at 52–64 °C (Annealing), 3.5 min at 72 °C (Extension), and then a 7 min of the final extension at 72 °C. The best annealing temperatures (producing the minimum number of unspecific alleles and minor stutter bands) were applied for each primer for the main PCR amplification (Table 2). The first series of the SSR primers (1–7) were designed by using Primer3 program [11], based on the Indian AP sequences obtained from the National Centre for Biotechnology Information (NCBI) (Table 2). These sequences belonged to the Indian AP population from Kerala state [9], as mentioned before these accessions might be the origin of the currently available populations of AP in Malaysia. The second series of the SSR primers (8–15) belonging to *Acanthus ilicifolius*, and (16–24) *Lumnitzera racemosa* [12] were cross-amplified in the Malaysian AP accessions (Table 2).

To make a precise genotyping, two types of gel including 4 % MetaPhor and 8 % Acrylamide gels were used in this experiment. 1× TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3) was used for both gels. The above-mentioned Acrylamide and MetaPhor gels were respectively subjected to the vertical and horizontal gel electrophoresis systems (Bio-Rad, USA). The gels were stained with Ethidium Bromide (0.5 μg/mL) and de-stained with distilled water. Banding profiles were visualized using a UV Gel Documenter (Bio-Rad, USA). The PCR reactions were repeated at least twice to test the reproducibility of each primer. The PCR product of each primer was sequenced after purification. Due to the small size of the PCR products, only reverse sequencing was conducted for each PCR product by sending them to two different labs. After processing the reverse complementary step, the sequence alignment was performed using BLAST (Basic Local Alignment Search Tool), MAFFT and CLUSTAL W online multiple alignment tools (www.ebi.ac.uk). The sequences of the Malaysian accessions were then compared with the Indian AP population from Kerala state located in the south of India [9].

Results

Apparently, due to the monomorphic profiles of the studied loci in AP, running any routine microsatellite analyses was out of context. Considering the mating system of the herb (self-pollinated), makes these observations to become more understandable. Besides, the sequence analysis revealed that there was no variation in the repeat motifs of the Malaysian AP accessions which were selected for this experiment. Despite the fact that no polymorphism was detected in the studied loci, the result of this investigation included a sort of completely novel and pivotal information about AP from evolutionary and phylogenetic point of views that could be presented in two different categories comprising (a) intra-specific (the primers 1–7) and (b) inter-specific or cross-species (the primers 8–24) amplification of microsatellites.

Intra-specific microsatellite amplification

Surprisingly, it was realized that the size and repeat motif of the microsatellites detected in Malaysian accessions using the same primers were totally different from the Indian AP accessions (Table 3, 4). In addition, the co-dominant nature of the microsatellite markers showed that the Malaysian accessions were heterozygous in loci Androg1, Androg2, Androg3 and Androg6, while they were found homozygous in loci Androg4 and Androg5. Furthermore, no band was generated by primer 1 in the Malaysian accessions. As a result, in this part of the study, six new microsatellite loci (Androg1–6) were generated, while the whole fragment size and the type of repeat motifs were totally different from the Indian AP accession. As another significant outcome, the "fixed heterozygosity" phenomenon was detected among the Malaysian AP accessions, in which all the heterozygous loci such as Androg1 (Table 3; Fig. 1a) showed no polymorphism at all. The same trend was observed in homozygous loci such as Androg5 (Table 3; Fig. 1b).

Inter-specific (cross-species) microsatellite amplification

This part of research was carried out by using the 17 primers belonging to two different species where it resulted in producing 10 novel microsatellite loci (Androg7–16) as well as re-occurrence of "fixed heterozygosity" phenomenon in Malaysian AP accessions with an emphasis on the amplification pattern of Androg8, Androg9, Androg10, Androg11, Androg12 and Androg15 loci (Table 4; Fig. 2a, d). Moreover, the primers 1, 8, 11, 12, 20, 21, 22 and 23 failed to amplify any microsatellite locus in AP.

Discussion

Fixed heterozygosity, genetic bottleneck and founder effect

A population or genetic bottleneck is an evolutionary event in which a significant proportion of a population or species is disappearing or otherwise prevented from reproducing. To address the evolutionary relationship between Malaysian and Indian AP, the obtained results could be debated through

Table 2 List of the SSR primers used in the present study

Primer	GenBank acc. no.	Locus	Locus name in Malaysian AP	Primer sequence (5′→3′)	T_a (°C)	SS (bp)
1	AY283927	NL	NL	F: GCAGTAGTGGCTCGTGATGA R: ACACCCCGACTTCTAGCAAC	NA	268
2	AY283926	NL	Androg1	F: TCATATCTGGTGCATACTACTTGTAAA R: GGTTGGGAGCTCTAGTGATTCT	57	190
3	AY283925	NL	Androg2	F: GAGCAGTAGAATGGGAGACGA R: CGACACCTTTTTAATCATTAGGG	58	187
4	AY283924	NL	Androg3	F: TCATGATAAAGTAGGGTTTTGAAGG R: GATTCTCAATCGGCTTGCTC	58	238
5	AY283923	NL	Androg4	F: GACTGAGTAAGCACACAGATGTAGA R: AGTAATCATCAGTTGTGCACTAAAGC	58	196
6	AY283922	NL	Androg5	F: GAGATCTGTCCGGTGTGCTC R: CCTCCTCTTTGTCACTCACTTCT	58	133
7	AY283921	NL	Androg6	F: CGTTCGATAAATCCACTGTGA R: GAAGAGTGAGTGTGTTTCTCTCCA	58	183
8	AB300577	Acai13	NL	F: ATAATTTATCATGGCCATCG R:AATGTGGAGAATTTGGCATCTGGTGCG	NA	152–180
9	AB300578	Acai02	Androg7	F: ACACACACACACTCTCTCTCTC R: CCATCTGAAAATAGATGGTAGGAC	63	205–217
10	AB300579	Acai08	Androg8	F : TCTCTCTCTCTCACACACACAC R: GAGGTACTAGGGTTTCAACATGAT	63	184–190
11	AB300580	Acai11	NL	F: TCTCTCTCTCTCACACACACAC R: CCCCCATATTTGTATATGTTTG	NA	128–134
12	AB300581	Acai12	NL	F: TCTCTCTCTCTCACACACACAC R: CCACCAATGGTGCTGAATACATTG	NA	87–95
13	AB300582	Acai13	Androg9	F: TCTCTCTCTCTCACACACACAC R: CCGACAAGCTCCTCCAGTATTTTG	63	127–129
14	AB300583	Acai14	Androg10	F: TCTCTCTCTCTCACACACACAC R: GCGTAGGTCGGATGTATGCAAAC	61	92–100
15	AB300584	Acai15	Androg11	F: TCTCTCTCTCTCACACACACAC R: CCACAAGAACCCACTTATAACTCA	56	95–99
16	AB300585	Lumr09	Androg12	F: TCTCTCTCTCTCTCACACAC R: AAAGGGCAAAGGGAAGAGGATTTC	58	58–60
17	AB300586	Lumr11	Androg13	F: TCTCTCTCTCTCTCACACAC R: TTGTGACGTAATAGCCACCCC	58	67–75
18	AB300587	Lumr14	Androg14	F: TCTCTCTCTCTCTCACACAC R: GCTTCTTTGCTTGCACATGTGAAG	58	84–86
19	AB300588	Lumr17	Androg15	F: TCTCTCTCTCTCTCACACAC R: CCCCTTCGATACCTAAATCAACCA	58	114–118
20	AB300589	Lumr19	NL	F: TCTCTCTCTCTCTCACACAC R: CCACCTTTGGATTTATGAAAGGTA	NA	179–181
21	AB300590	Lumr26	NL	F: TCTCTCTCTCTCTCACACAC R: ACAAGGGGTTAGAGGTTAGGAGTG	NA	158–162
22	AB300591	Lumr28	NL	F: ACACACACACACACAGAGAG R: GGCTTTTCAATCTTATCCGAAGTT	NA	194–238
23	AB300592	Lumr29	NL	F: ACACACACACACACAGAGAG R: CCTGGGATTGCATTTGAGCTGCAA	NA	131–153
24	AB300593	Lumr30	Androg16	F: TCTCTCTCTCTCTCACACAC R: CAACCGTACTACGAATGCCAAGTC	60	106–108

T_a annealing temperature used in the present study, *SS* sequence size of the fragments in original plants, *NL* nameless, *NA* non amplified, *F* forward primer, *R* reverse primer, *Androg* SSR loci amplified in the Malaysian *A. paniculata* accessions, *Acai* SSR loci amplified in *A. ilicifolius*, *Lumr* SSR loci amplified in *L. racemosa* [12]

Table 3 Comparison of the SSR repeat motifs and allele size between the Malaysian and Indian AP accessions

Primer	GenBank acc. no.	Repeat motif of Indian AP	No. of alleles	Allele size (bp)	Locus	Repeat motif of Malaysian AP	No. of alleles	Allele size (bp)
1	AY283927	$-(AT)_8-(GA)_{30}-$	1	171	NL	NA	NA	NA
2	AY283926	$-(AG)_{13}-$	1	175	Androg1	$-(CT)_{10}-$	2	176–276
3	AY283925	$-(TC)_{23}-$	1	151	Androg2	$-(GA)_{22}-$	2	152–172
4	AY283924	$-(AT)_9-(AG)_{13}-$	1	151	Androg3	$-(AG)_{11}-$	2	244–388
5	AY283923	$-(AG)_{18}-$	1	150	Androg4	$-(AG)_{10}-$	1	170
6	AY283922	$-(GAA)_5(GATA)_5-$	1	100	Androg5	$[-(GAG)_3-]_4-G_7C_5G_1C_5G_1-$	1	277
7	AY283921	$-(ATAG)_5-$	1	181	Androg6	$-(AT)_{15}-$	2	200–320

Androg SSR loci amplified in the Malaysian *A. paniculata* accessions of the present study, *NL* nameless, *NA* non amplified

different angles. In this regard, the monomorphic banding pattern of the heterozygous alleles (in Androg1, Androg2, Androg3, Androg6, Androg8, Androg9, Androg10, Androg11, Androg12 and Androg15 loci) complied with the occurrence of the fixed heterozygosity phenomenon in the herb (Tables 3, 4). A slightly different sort of genetic bottleneck can occur if a small group becomes re-productively separated from the main population and this is called a founder effect. This model is in agreement with the idea that suggests the founder effect produce the genetic bottleneck [13]. This can be evidenced by the changes in type and length of the repeat motifs in Malaysian AP population. Plus, the recent introduction of AP into Malaysia and its self-pollinating mating system are the intensification factors in this case [2, 14]. Hence, if we accept that AP has been introduced to Malaysia almost 200 years ago (and onward), then providing proof to the incidents such as founder effect as well as the impacts of domestication and intensive artificial selections on the Malaysian AP populations should not be difficult.

Despite the above-mentioned facts, other mutagenic actions cannot be pulled out. For instance, a flanking small insertion [15] of the locus Androg4 was accompanied with a partial deletion in the repeat motif of the same locus, while a complete deletion occurred in the locus AY283927 (Table 3).

Nonetheless, our results do not prove the Malaysian AP as an independent species, but we are convinced that the Malaysian AP has been descended from a core Indian AP population.

Fixed heterozygosity of the Malaysian AP could be caused by the ecological bottleneck that probably happened after the mentioned immigration from India. On the other hand, there is a possibility that after AP was brought into Malaysia, it tried to adapt itself to the new environment by duplicating some parts of its genome [16]. This is what actually happened in loci Androg2 and Androg6, where the same alleles (with the same repeat motifs) have been repeated twice (i.e., signature of genetic bottleneck) just with a difference in size (Table 3).

Most bottlenecks are the result of domestication, migration, or environmental disease factors occurring in the distant past. The duration and number of individuals are mostly unknown and can only be inferred from molecular data [17].

Low biodiversity and population disconnection

Low genetic diversity in AP is not a new issue. As an analogous clue, SSR markers revealed a low expected heterozygosity due to the low number of polymorphic bands in AP population in Thailand [33]. Disregarding the stated environmental changes, it has been affirmed that disconnection from the original population can create the genetic bottleneck. The explanation of such a consequence is that population disconnection and the driven bottleneck depresses the allelic richness and gene diversity (heterozygosity) [18]. Therefore, the population's ability to adapt to new selective pressures, such as climatic change or shift in available resources is declining. As a population becomes smaller, genetic drift plays a bigger role in speciation. Genetic drift can eliminate alleles that could have been positively selected on by the environment if they had not already drifted out of the population [19]. In tune with Francisco's opinion [20], the locus Androg5 as an interrupted microsatellite showed a higher stability by producing a single allele in the studied accessions [20]. This locus can be used as a molecular indicator to determine the location of the fragment in chloroplast, because it contains a single nucleotide repeat motif which typically fits a chloroplast microsatellite or so-called cpSSR (Table 3).

When a population goes through a bottleneck, the genetic variability is expected to decline rapidly, and this principle can be used as a logical explanation to justify the no-diversity situation in the Malaysian AP population. Moreover, in the absence of selection (i.e. if alleles are selectively neutral), migration homogenizes allele frequencies among populations. On the other hand, a population bottleneck occurs when the effective population size

Table 4 Comparison of the SSR repeat motifs and allele size in *A. paniculata*, *A. ilicifolius*, and *L. racemosa*

Primer	Locus	Repeat motifs of *A. ilicifolius* & *L. racemosa*	No. of alleles	Allele size (bp)	Locus	Repeat motifs of Malaysian *A. paniculata*	No. of alleles	Allele size (bp)
8	Acai13	$(TC)_8(AC)_{11}$	8	152–180	NL	NA	NA	NA
9	Acai02	$(AC)_6(TC)_{10}$	3	205–217	Androg7	$–(GA)_{14}–(GGGT)_3–$	1	330
10	Acai08	$(TC)_6(AC)_5$	4	184–190	Androg8	$–(CA)_6–T_3A_1T_{11}$ & $–(TC)_5–T–(AC)_2–(AT)_2–(AC)_4–$	2	144–290
11	Acai11	$(TC)_6(AC)_{12}$	3	128–134	NL	NA	NA	NA
12	Acai12	$(TC)_6(AC)_{10}$	5	87–95	NL	NA	NA	NA
13	Acai13	$(TC)_6(AC)_6$	2	127–129	Androg9	$–(TC)_6–(AC)_6–$ & $–(TC)_6–(AC)_{12}–$	2	266–336
14	Acai14	$(TC)_6(AC)_{13}$	4	92–100	Androg10	$–(TC)_6–(AC)_6–$ & $–(AC)_5–(TC)_6–(AC)_7–$ & $–(TC)_7–(AC)_{11}–$	3	150–200– 244
15	Acai15	$(TC)_6(AC)_{10}$	3	95–99	Androg11	$–(TC)_7–(AC)_5–$ & $–(TC)_6–(AC)_6–$ & $–(TC)_4–(AC)_{10}–$	3	90–120–178
16	Lumr09	$(TC)_6(AC)_8$	2	58–60	Androg12	$–(TC)_9–(AC)_3–$ & $–(TC)_7–(AC)_8–$	2	90–128
17	Lumr11	$(TC)_6(AC)_6$	4	67–75	Androg13	$–(TC)_{10}–(AC)_{10}–$	1	177
18	Lumr14	$(TC)_6(AC)_5$	2	84–86	Androg14	$–(TC)_6–(AC)_{11}–$	1	180
19	Lumr17	$(TC)_6(AC)_{15}$	3	114–118	Androg15	$–(TC)_8–(AC)_8–$ &$–(TC)_8–(AC)_4–$	2	230–344
20	Lumr19	$(TC)_6(AC)_{11}$	2	179–181	NL	NA	NA	NA
21	Lumr26	$(TC)_6(AC)_8$	3	158–162	NL	NA	NA	NA
22	Lumr28	$(AC)_6(AG)_{18}$	9	194–238	NL	NA	NA	NA
23	Lumr29	$(AC)_6(AG)_{12}$	4	131–153	NL	NA	NA	NA
24	Lumr30	$(TC)_6(AC)_7$	2	106–108	Androg16	$–(TC)_8–(AC)_9–$	1	330

Acai SSR loci amplified in *A. ilicifolius*, *Lumr* SSR loci amplified in *L. racemosa* [12]. *Androg* SSR loci amplified in the Malaysian *A. paniculata* accessions, during the present study, *NL* nameless, *NA* non amplified

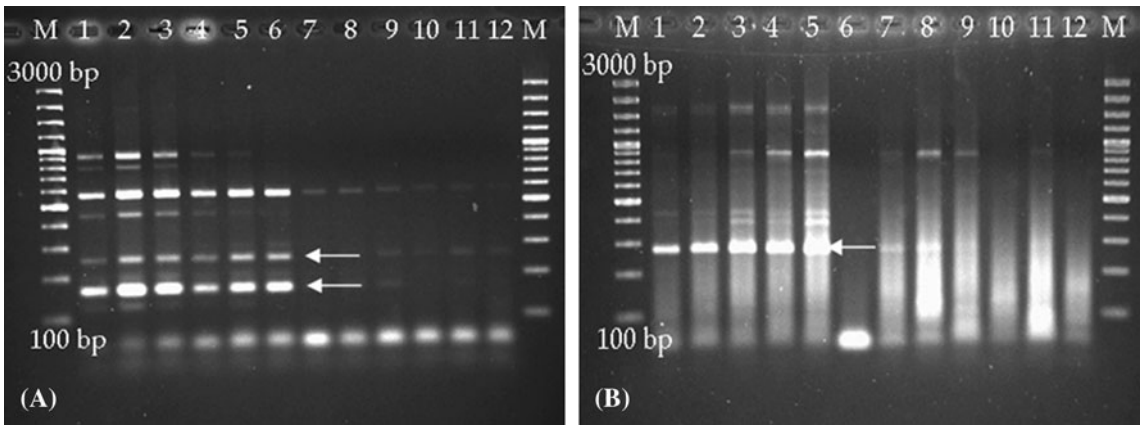

Fig. 1 SSR-Gradient PCR gel images of Malaysian AP accession generated by SSR primers: **a** Primer 2, **b** Primer 6, on MetaPhor gel 4 %. The *codes 1–12* represent the annealing temperatures including 52, 52.3, 53.2, 54.4, 55.8, 57.2, 58.8, 60.2, 61.6, 62.8, 63.7 and 64 °C, respectively and (M) 100 bp Fermentas size marker. The arrows show the target alleles which are containing the SSR repeat motif. The bands without arrow can be considered as so-called unspecific that based on sequencing results, did not contain any repeat motif

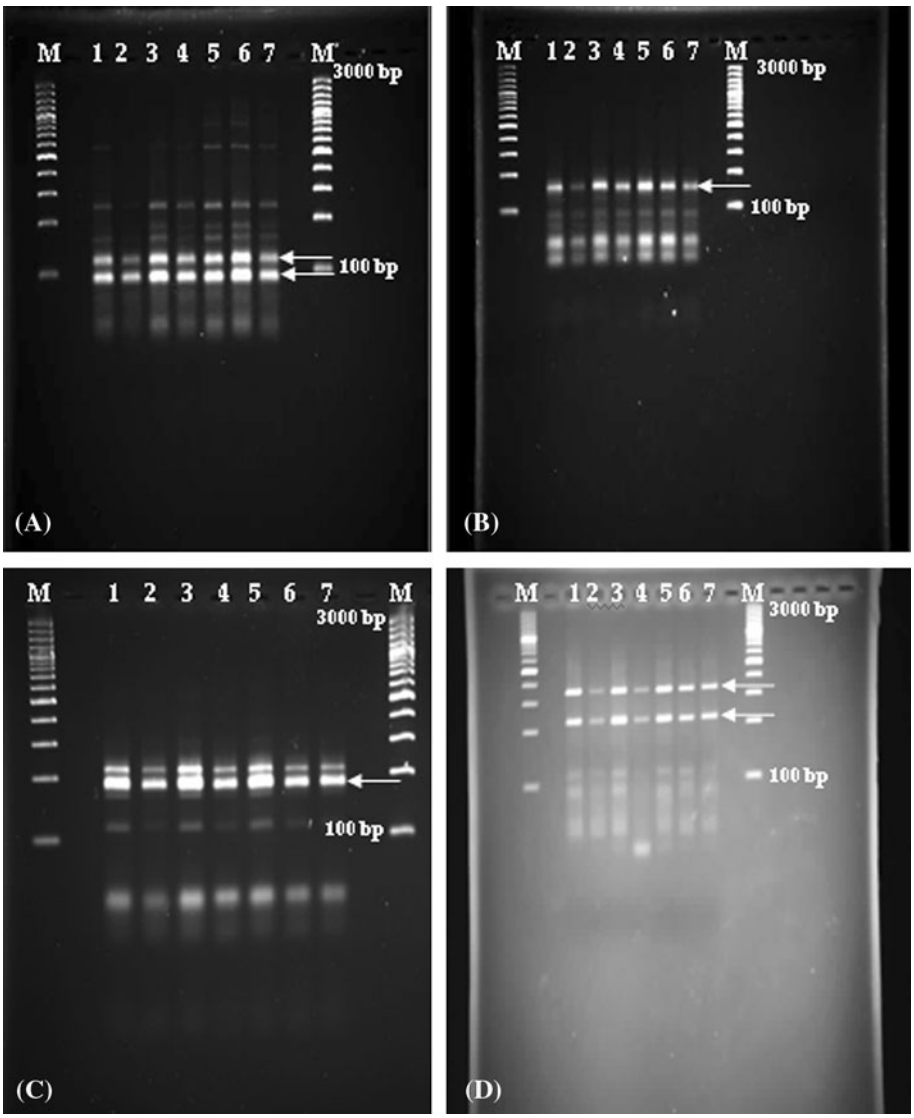

Fig. 2 Monomorphic DNA fragments of seven AP accession generated by SSR primers; **a** 16 N, **b** 17 N, **c** 18 N, **d** 19 N on MetaPhor gel 4 %. *Lanes 1–7* represent the accessions and (M) 100 bp Fermentas size marker. The *arrows* show the target alleles which are containing the SSR motif repeats. The bands without arrow are considered unspecific bands and stutters that were not containing none motif repeat (based on sequencing results)

(N_e) sharply decreases to a small percentage of the initial amount. In the short-term, immediate impact of a population bottleneck is to reduce genetic diversity, promoting the effects of stochastic genetic drift over natural selection. In the long-term, frequentative population bottlenecks can critically decrease population fitness. Deleterious alleles are able to accumulate especially where the time gap between bottlenecks prevents the generation of new alleles via mutation. Once the above-mentioned factors are linked to the mating system of the herb as well as to the over-exploitation issue, then serious debates about conservation of the species arise in the country. This is precisely has been highlighted in a very recently conducted study, where the intra-specific hybridization between the same Malaysian AP accessions boosted the genetic variation and prompted a heterosis in most of the agro-morphological traits of the species [4].

Speciation or mutation?

Even though in general on the basis of polymorphism, microsatellite analysis could conclude whether bottleneck has been operated in a population, the absence of such a polymorphism in the present study does not prevent the verdict of bottleneck occurrence in the Malaysian AP population. Anyway, the lack of polymorphism makes us unable to determine whether the Malaysian AP population's bottleneck underwent an infinite allele mutation model (IAM) or a stepwise mutation model.

However, observing such an extremely low genetic diversity or better to say "no genetic diversity" leads us towards the most probable theory, in which the bottleneck happened immediately after AP was introduced to Malaysia due to climatic changes or environmental stresses, and the bottlenecked AP population tried to survive up to now. This situation supports the hypothesis that the Malaysian AP has been descended from a limited-original AP population from India or Sri Lanka. Nevertheless, the morphological differentiations of the available AP accessions in Malaysia could be attributed to epigenetic dissimilarities.

As a matter of experience, bottlenecks also play an important role in speciation and founding events [21, 22], but about AP, the morphological similarities between the Indian and Malaysian accessions are more than that to allow us to introduce the Malaysian AP as a new species completely separated from its Indian ancestors. On the other hand, as a general principle, there is a negative relationship between the bottleneck and mutation (frequentative population bottlenecks decrease the positive mutation). This means that the Malaysian AP has not been subjected to genetic bottleneck frequently, because if it had happened, then the mutagenic phenomena would not have been detectable in the microsatellite loci. Therefore, the occurrence of mutation seems more feasible than speciation in AP.

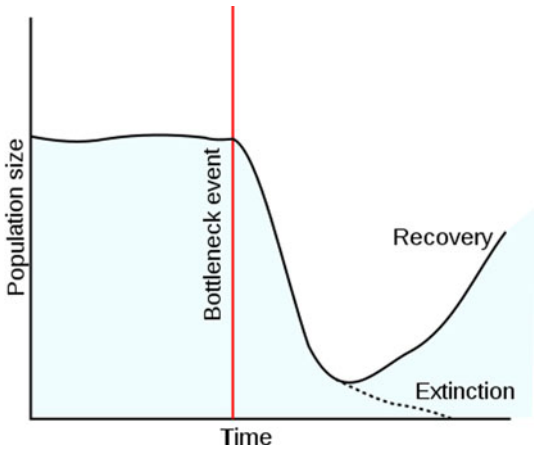

Fig. 3 Graphical demonstration of the genetic bottleneck (Source: Wikimedia)

Population bottleneck; recovery or extinction?

As cited by Ramakrishnan et al. [23], genetic bottlenecks enhance the rate of random genetic drift [24] and increase inbreeding [25] leading to the loss of genetic variation, e.g. as documented in [26] and [27], which may lead to lower levels of individual fitness [28], reduced resistance to parasites and diseases, and reduced ability to respond to environmental change [29, 30]. All these will end up with a vague feature in the conservation of AP. Figure 3, demonstrates the two possible trends resulted by the genetic bottleneck in different populations. However, it is difficult to predict which tendency will be followed by AP in Malaysia, but the recovery of the herb under current circumstance is seemingly out of reach.

Evolutionary connectedness and conservation issues

The outcomes of the present study could be argued from two different points of view (1) to introduce the species as a cryptic species while it is genetically different from its Indian ancestors or (2) to be more concerned about it as an endangered species. Previously, AP has been declared as an endangered species in India and Sri Lanka [31, 32]. Taking these experiences into account along with our molecular observations, draw a red line, which tends to a critical condition indicating the threatening situation of AP in Peninsular Malaysia.

Nevertheless, our results need to be confirmed by applying extra sets of microsatellite markers, while developing microsatellites for this species should be stressed as a research priority at the first step. In the meanwhile, intra and inter population hybridization (out-

crossing) programs [4], as well as non-classical breeding strategies are suggested to improve the current deficiency.

All in all, the present achievement will serve as a reference for the future studies on the AP genome. Analysis of a large number of AP accessions from across the globe using extra microsatellites should be done to ascertain the validity of this hypothesis. The shotgun sequencing (cloning) approach as well as the next generation sequencing assay can be combined with ultra-accurate DNA polymerase such as Pfu Taq to produce more trusted results. It should be reminded that the Pfu Taq was applied to the two randomly selected DNA samples and also two primers of this study, while the Dream Taq's results were re-confirmed and no polymorphism was detected.

As an undeniable fact, the science is progressing quickly. Sooner or later, our results can be asserted and vice versa. Nonetheless, at the time being, it is obvious that the King of Bitters (Acı Paşa or Hempedu Bumi) holds two distinctive faces; a simple exterior (phenotype) versus a complex interior (genotype), like its other counterparts.

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