



# Analysis of genetic diversity and population structure in Cambodian melon landraces using molecular markers

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**Abstract** Genetic diversity of Cambodian melons was evaluated by the analysis of 12 random amplified polymorphic DNA (RAPD) and 7 simple sequence repeat (SSR) markers using 62 accessions of melon landraces and compared with 231 accessions from other areas for genetic characterization of Cambodian melons. Among 62 accessions, 56 accessions were morphologically classified as small-seed type with seed lengths shorter than 9 mm, as in the horticultural groups Conomon and Makuwa. Gene diversity of Cambodian melons was 0.228, which was equivalent to those of the groups Conomon and Makuwa and smaller than those of Vietnamese and Central Asian landraces. A phylogenetic tree constructed from a genetic distance matrix classified 293 accessions into three major clusters. Small-seed type accessions from East and Southeast Asia formed clusters I and

II, which were distantly related with cluster III consisting of large-seed type melon from other areas. All Cambodian melons belonged to cluster I (except three accessions) along with those from Thailand, Myanmar, Yunnan (China), and Vietnam (“Dua thom” in the northwest), thus indicating genetic similarity in these areas. In addition, the Cambodian melons were not differentiated among geographical populations. Conomon and Makuwa were classified into cluster II, together with melon groups from the plains of Vietnam. The presence of two groups of melons in Southeast Asia was also indicated by population structure and principal coordinate analysis. These results indicated a close genetic relationship between Cambodia and the neighboring countries, thus suggesting that Cambodian melons are not directly related to the establishment of Conomon and Makuwa.

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## Introduction

Reflecting its long history of cultivation, various usage, and adaptation in various parts of the world, melon (*Cucumis melo*) is known as the most diversified among Cucurbitaceae crops. Indeed, great diversity is reported in its morphological traits, such as fruit weight and seed length ranging from 50 to 15 kg and 4.5 to 15.0 mm, respectively (Nuñez-Palenius et al. 2008; Akashi et al. 2002). *C. melo* is divided into 19 horticultural groups based primarily on flower and fruit traits: Agrestis, Kachri, Chito, Tibish, Acidulus, Momordica, Conomon, Makuwa, Chinen-sis, Flexuosus, Chate, Dudaim, Chandalak, Indicus, Ameri, Cassaba, Ibericus, Inodorus, and Cantalupensis (Pitrat 2016).

Fujishita and Nakagawa (1973), focusing on seed size variation, indicated that melon is classified into large-seed ( $\geq 9.0$  mm) and small-seed ( $< 9.0$  mm) types. Melon accessions of groups Cantalupensis and Inodorous are mostly classified as large-seed type and groups Agrestis, Conomon, and Makuwa as small-seed type according to Fujishita (1983) and Akashi et al. (2002). In Asia, groups Dudaim, Flexuosus, Chandalak, Ameri and Inodorus of large-seed type are mainly distributed in West Asia, Central Asia and India, whereas groups Chinen-sis, Conomon, Makuwa and Acidulus of small-seed type are distributed in Southeast and Far-East Asia and India (Stepansky et al. 1999; Akashi et al. 2002; Yashiro et al. 2005; Tanaka et al. 2007; Pitrat 2008; Tanaka et al. 2013).

Among Asian melons, small- and large-seed types have proven to be diversified in independent maternal lineages, Ia and Ib, respectively, according to the analysis of chloroplast genome (Tanaka et al. 2013). India and Africa are now considered as domestication centers of melon (John et al. 2013; Endl et al. 2018; Gonzalo et al. 2019), where small- and large-seed types and Ia and Ib types are frequently distributed. However, the landraces of Conomon and Makuwa are not reported in India. Conomon and Makuwa are mostly distributed from China to Japan and are considered to be diversified in small-seed melon with Ia type cytoplasm in areas somewhere lying between India and China (Akashi

et al. 2002; Tanaka et al. 2007; Serres-Giardi and Dogimont 2012). In contrast to India and Far-East Asia, little is known about melon landraces in Southeast Asia.

It was proven that Myanmar melon landraces first investigated by Yi et al. (2009) possess large genetic variation as in Indian melon. Myanmar melons consist of both small- and large-seed types, and Yi et al. (2009) classified small-seed type accessions as Conomon, Momordica, or Agrestis. However, most Myanmar Conomon accessions were clustered separately from the Conomon and Makuwa of Far-East Asia. Although one accession was regarded as Makuwa, they could not rule out the possibility of its recent introduction from other countries such as China and Japan.

More recently, Nhi et al. (2010) and Duong et al. (2021) analyzing Vietnamese melon landraces showed that all except one accession were small-seed type. Vietnamese melon consists of seven cultivar groups, including “Dua le” and “Dua vang” regarded as Makuwa and “Dua bo” and “Dua gang-andromonoecious” as Conomon. In contrast, “Dua thom” and “Montok” showed genetic similarities with Indian and Myanmar landraces. Based on these results, the presence of groups Conomon and Makuwa was first confirmed in Vietnam. Therefore, to identify the origin of groups Conomon and Makuwa, melon landraces of Cambodia, Laos, and Thailand should be investigated.

We have conducted germplasm collection expeditions in Cambodia since 2014 and successfully introduced germplasms of melon landraces (Matsunaga et al. 2015; Tanaka et al. 2016, 2017, 2019). Genetic resources of Cambodian melon are not available in major Genebanks other than NARO Genebank, National Agriculture and Food Research Organization, Japan, so they are expected to contribute to breeding for disease resistance and to the analysis of diversification of melon in Asia. More specifically, genetic analysis of Cambodian landraces is essential for understanding the distribution of groups Conomon and Makuwa and their genetic relationships with Vietnamese landraces. Therefore, in this study, we aimed to identify the genetic diversity and genetic structure of Cambodian melons using RAPD and SSR markers, and to discuss genetic diversification in Asian melons.

## Materials and methods

### Plant materials

Among the Cambodian melon landraces collected during 2014–2017, 62 accessions were selected to cover most of Cambodia and divided into five geographical groups: east, center, west, north, and south. Details of the materials used are summarized in Table 1 and Fig. 1. These accessions have been introduced to NARO Genebank, Japan, with SMTA (Standard Material Transfer Agreement). A total of 229 accessions were used as references, including Conomon (9), Makuwa (11), Agrestis (6), Cantalupensis (10), Inodorus (8), and accessions from different areas (Vietnam: 58, Myanmar: 36, Thailand: 5, Yunnan (China): 5, Xinjiang (China): 24, Central Asia: 14, Pakistan: 11, Afghanistan: 9, Iran: 10, Spain: 8, and the USA: 5). Two accessions of wild cucumber *C. sativus* var. *hardwickii* were also used as the outgroup. Seed length and width were measured for 10 seeds of each accession, and accessions were classified as large-seed ( $\geq 9.0$  mm in length) or small-seed ( $< 9.0$  mm in length) types, according to Akashi et al. (2002).

### DNA extraction

Seeds of each accession were germinated on wet filter paper in a Petri dish and later transferred into pots, and grown in an incubator maintained at 30 °C with a 16 h light–8 h dark cycle at a light intensity of  $46.5 \mu\text{Ms}^{-1}\text{-m}^{-2}$ . After 2 weeks, cotyledons from one seedling of each accession were ground individually in the liquid nitrogen. Total DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) method (Murray and Thompson 1980) with minor modifications. The quality and quantity of each DNA sample were determined with a spectrophotometer.

### RAPD analysis

Ten random primers which were selected for their ability to detect polymorphism and for their stability in PCR amplification were used for RAPD analysis according to Nhi et al. (2010) (Table 2). PCR amplification was carried out in a 10  $\mu\text{l}$  mixture containing 50 ng genomic DNA, 1  $\mu\text{l}$  PCR buffer (Sigma®, St. Louis, MO, USA: 10 mM Tris–HCl; pH 8.3, 50 mM

KCl), 2.5 mM  $\text{MgCl}_2$ , 0.1 mM dNTP, 0.5  $\mu\text{M}$  primer, and 0.25 U *Taq* polymerase (Sigma) by using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR cycle was started with an initial denaturing step at 95 °C for 3 min, 40 cycles at 93 °C for 1 min, 40 °C for 2 min, and 72 °C for 2 min. The duration of annealing temperature at 40 °C and the duration of extension at 72 °C were modified to 45 s and 1.5 min, respectively, for three primers A31, A57 and B86. The final extension step was at 72 °C for 5 min for all primers. After amplification, samples were electrophoresed on a 1.5% agarose gel (GenePure LE, BM Bio, Tokyo, Japan) at a constant voltage of 100 V using a horizontal gel electrophoresis system (Mupid-2, Cosmo Bio, Tokyo, Japan). Then, the PCR products were visualized with ethidium bromide staining, and their polymorphisms were evaluated.

### SSR analysis

Seven SSR markers, developed by Ritschel et al. (2004) and Fukino et al. (2007) and showing high polymorphism in Vietnamese landraces as selected by Nhi et al. (2010), were used for the analysis of Cambodian melon landraces (Table 2). The PCR mixture was the same as used in the RAPD analysis, but the primer concentration was changed to 0.25  $\mu\text{M}$  each. The PCR cycle was started with an initial denaturing step at 95 °C for 3 min, 35 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The final extension step was at 72 °C for 5 min. PCR products were electrophoresed on 10% nondenatured polyacrylamide gel at a constant voltage of 260 V. Gels were stained in the same way as for RAPD analysis, and the size of the smallest band of each landrace was compared among the landraces.

### Genotyping of *CmACS7*

The *CmACS7* genotype was determined for sex expression analysis using the Cleaved Amplified Polymorphic Sequences (CAPS) marker developed by Boualem et al. (2008). The details of the PCR condition were given in Duong et al. (2021).

### Data analysis

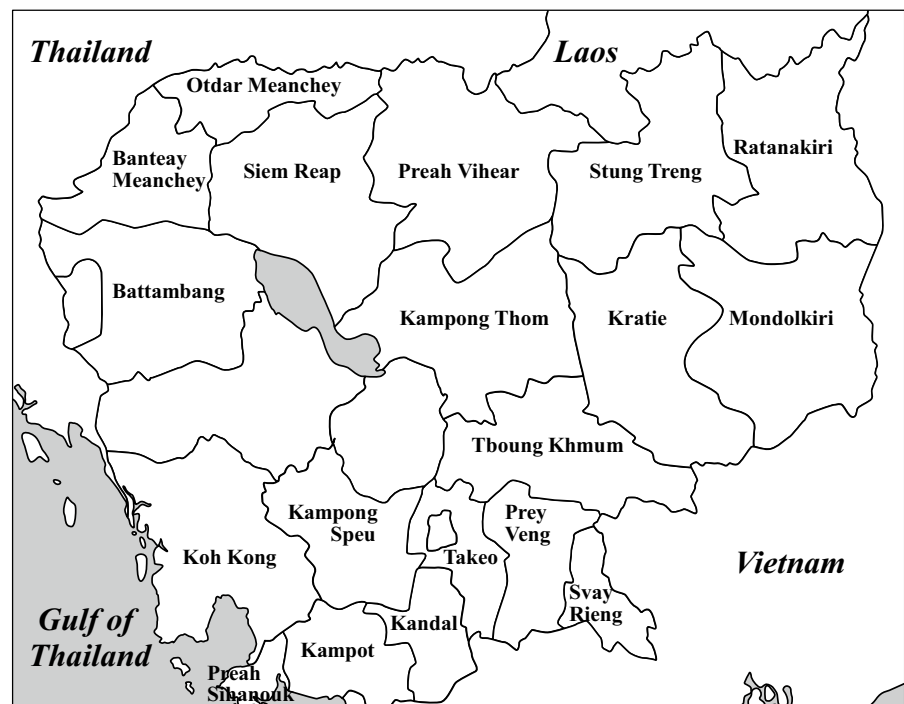
DNA fragments were scored as present (1) or absent (0) for RAPD markers. For SSR, DNA fragments

**Table 1** List of Cambodian melon landraces analyzed in this study

Accession No.	Collection No.	Province	Region	Year of collection	Seed size	Seed length (mm)	Cluster	Population
CAe 1	15CJV-C83-1	Ratanakiri	East	2015	Small	8.1	I a	P2
CAe 2	15CJV-C100	Ratanakiri	East	2015	Small	7.7	I d	P2
CAe 3	15CJV-C94-1	Ratanakiri	East	2015	Small	8.2	I d	P2
CAe 4	15CJV-C94-2	Ratanakiri	East	2015	Small	7.0	I a	P2
CAe 5	15CJV-C76	Mondolkiri	East	2015	Small	8.3	I a	P2
CAe 6	15CJV-C40-1	Mondolkiri	East	2015	Large	9.4	I a	P2
CAe 7	15CJV-C40-2	Mondolkiri	East	2015	Small	7.5	I a	P2
CAe 8	15CJV-C19	Mondolkiri	East	2015	Small	7.3	I d	P2
CAe 9	15CJV-C132-1	Stung Treng	East	2015	Small	7.9	I d	P2
CAe 10	15CJV-C132-2	Stung Treng	East	2015	Small	6.3	I d	P2
CAe 11	15CJV-C138	Stung Treng	East	2015	Small	8.6	I d	P2
CAe 12	15CJV-C139	Stung Treng	East	2015	Small	8.2	I d	P2
CAe 13	16CJVC-83	Stung Treng	East	2016	Small	7.6	I d	P2
CAe 14	16CJVC-107	Kratie	East	2016	Small	7.7	I c	P2
CAe 15	16CJVC-107	Kratie	East	2016	Small	6.7	III a	P2
CAC 16	16CJVC-19	Kampong Thom	Center	2016	Small	8.6	I d	P2
CAC 17	16CJVC-19	Kampong Thom	Center	2016	Small	7.3	I d	P2
CAC 18	16CJVC-11-1	Kampong Thom	Center	2016	Small	8.6	I d	P2
CAC 19	16CJVC-11-2	Kampong Thom	Center	2016	Small	7.4	I d	P2
CAC 20	15CJV-C9	Tboung Khmum	Center	2015	Small	8.6	I a	P2
CAw 23	58	Battambang	West	2014	Small	8.2	I a	P2
CAw 24	65	Battambang	West	2014	Small	7.9	I a	P2
CAw 25	68	Battambang	West	2014	Large	9.0	I e	P2
CAw 26	68	Battambang	West	2014	Small	7.1	I d	P2
CAw 27	78	Banteay Meanchey	West	2014	Small	7.6	I c	P2
CAw 28	16CJVC-52	Banteay Meanchey	West	2016	Small	8.5	I c	P2
CAw 29	16CJVC-52	Banteay Meanchey	West	2016	Small	6.6	I c	P2
CAn 30	16CJVC-72	Preah Vihear	North	2016	Small	8.5	I d	P2
CAn 31	16CJVC-72	Preah Vihear	North	2016	Small	7.2	I a	P2
CAn 32	16CJVC-74	Preah Vihear	North	2016	Large	9.3	I a	P2
CAn 33	16CJVC-74	Preah Vihear	North	2016	Small	7.6	I a	P2
CAn 34	16CJVC-80	Preah Vihear	North	2016	Small	8.4	I d	P2
CAn 35	16CJVC-80	Preah Vihear	North	2016	Small	7.1	I d	P2
CAn 36	16CJVC-100	Preah Vihear	North	2016	Large	9.1	I a	P2
CAn 37	16CJVC-100	Preah Vihear	North	2016	Small	8.2	I a	P2
CAn 38	16JCVC-16	Siem Reap	North	2016	Small	8.1	I c	P2
CAn 39	16JCVC-23	Siem Reap	North	2016	Small	7.8	I e	P2
CAn 40	16JCVC-37	Siem Reap	North	2016	Small	7.6	I c	P2
CAn 41	16JCVC-60	Siem Reap	North	2016	Small	7.7	I c	P2
CAn 42	16JCVC-60	Siem Reap	North	2016	Small	6.9	I d	P2
CAn 43	16CJVC-46	Oddar Meanchey	North	2016	Large	9.3	I d	P2
CAn 44	16CJVC-46	Oddar Meanchey	North	2016	Small	7.7	I a	P2
CAn 45	16CJVC-63	Oddar Meanchey	North	2016	Small	8.0	I a	P2
CAs 46	17CJVC-4	Svay Rieng	South	2017	Small	6.9	III a	Mix

**Table 1** (continued)

Accession No.	Collection No.	Province	Region	Year of collection	Seed size	Seed length (mm)	Cluster	Population
CAs 47	17CJVC-11	Svay Rieng	South	2017	Small	8.5	I a	P2
CAs 48	17CJVC-19-1	Prey Veng	South	2017	Small	8.5	I a	P2
CAs 49	17CJVC-19-2	Prey Veng	South	2017	Small	7.4	I d	P2
CAs 51	17CJVC-28	Takeo	South	2017	Small	7.7	I d	P2
CAs 52	17CJVC-48-1	Takeo	South	2017	Small	7.5	I a	P2
CAs 53	17CJVC-48-2	Takeo	South	2017	Small	6.6	II a	P2
CAs 54	17CJVC-87-1	Kompong Speu	South	2017	Large	9.5	I a	P2
CAs 55	17CJVC-87-2	Kompong Speu	South	2017	Small	8.3	I a	P2
CAs 56	17CJVC-87-3	Kompong Speu	South	2017	Small	7.3	I a	P2
CAs 57	17CJVC-92	Kompong Speu	South	2017	Small	7.9	I d	P2
CAs 58	17CJVC-52	Kampot	South	2017	Small	8.0	I a	P2
CAs 59	17CJVC-71	Koh Kong	South	2017	Small	7.7	I a	P2
CAs 60	17CJVC-64	Preah Sihanok	South	2017	Small	8.7	I a	P2
CAs 61	17CJVC-98	Kandal	South	2017	Small	4.9	I e	P2
CAs 62	17CJVC-101	Prey Veng	South	2017	Small	5.5	I e	P2
CAs 63	17CJVC-117	Kandal	South	2017	Small	5.1	I e	P2
CAs 64	17CJVC-121	Kandal	South	2017	Small	4.7	I e	P2
CAs 65	17CJVC-126	Kandal	South	2017	Small	4.7	I e	P2

**Fig. 1** Map of Cambodia showing 19 provinces where melon landraces were collected

**Table 2** Primer sequence of 10 random amplified polymorphic DNA (RAPD) and seven simple sequence repeat (SSR) markers used in this study and the size of polymorphic fragments

Marker	Primer sequence	Size of RAPD amplicon (bp)	No. of alleles per locus	PIC
<i>RAPD</i>				
A20	TTGCCGGGACCA	1100	2	0.353
		800	2	0.364
A22	TCCAAGCTACCA	1520	2	0.216
A31	GGTGGTGGTATC	800	2	0.338
A41	TGGTACGGTATA	930	2	0.162
A57	ATCATTGGCGAA	800	2	0.364
B32	ATCATCGTACGT	900	2	0.447
		700	2	0.047
B68	CACACTCGTCAT	1078	2	0.330
B71	GGACCTCCATCG	1220	2	0.426
B86	ATCGAGCGAACG	1350	2	0.066
B99	TTCTGCTCGAAA	1400	2	0.452
<i>SSR</i>				
CMBR2	F: TGCAAATATTGTGAAGGCGA R: ATCCCCACTTGTGGTTTG	114	12	0.891
CMBR83	F: CGGACAAATCCCTCTCTGAA R: GAACAAGCAGCCAAAGACG	142	9	0.825
CMBR120	F: CTGGCCCCCTCCTAAACTAA R: CAAAAAGCATCAAAATGGTTG	167	6	0.699
CMN04-03	F: ATCACAGAGACCGCCAAAAC R: GGTTGAAGATTGCGCTTGAT	218	9	0.829
CMN04-07	F: GAAAGCATTAAATATGGCATTGG R: AAGCTTAACAGCTTCCAGGG	286	8	0.664
CMN04-40	F: CACCTGACGATAGGGGTGTT R: AGTATTCGGGTTGGCAAAAA	212	9	0.795
CMN61-44	F: TGTGGAGTTTAATGAGGAAGGA R: AGAGAAGATGAATGGGGCAC	233	17	0.854

were scored based on their size from the smallest (1) to the largest. From these data, the polymorphic information content (PIC) was calculated following Botstein et al. (1980). Genetic similarity (GS) was calculated after Apostol et al. (1993), using the formula  $GS = (N11 + N00)/T$ , where N11 and N00 were the number of positive and null bands, respectively, shared between two accessions and T was the total number of bands scored. The genetic distance (GD) between them was calculated using the formula  $GD = 1 - GS$ . Gene diversity (D) within each group and genetic distance (GD) between each group were calculated according to Weir (1996) and Nei (1972),

respectively. A dendrogram was constructed by PHYLIP version 3.5.c (Felsenstein 1993), based on the GD matrix, with the unweighted pair group with arithmetic mean (UPGMA) method. Principal coordinate analysis (PCO; Gower 1966) based on the GS matrix was performed to show multiple dimensions of each population in a scatter-plot.

The population structure and the degree of admixture were estimated using the Bayesian clustering procedure of the STRUCTURE 2.3.4 (Pritchard et al. 2000). First the program was run to assume the number of distinct populations defined as K using the admixture model with correlated allele frequencies.

The parameter sets were configured as follows: the length of the burn-in period and the number of Markov chain Monte Carlo (MCMC) repetitions after burn-in were 10,000, and 50,000, respectively, for each  $K$  ranging from 1 to 10 and running 20 iterations. The most plausible number of clusters was determined in the Structure Harvester program (Earl and von Holdt 2012) by calculating the distribution of the  $\Delta K$  statistics as described by Evanno et al. (2005) and the performance of cluster matching across different  $K$  values was implemented through Cluster Markov Packager Across  $K$  (CLUMPAK) (Kopelman et al. 2015).

## Results

### Characterization of Cambodian melon landraces

Most Cambodian melons (56/62) were small-seed type as in those from Yunnan (China), Myanmar, Vietnam, Conomon, Makuwa and Agrestis (Table 3). In contrast, large-seed type was common in areas from Xinjiang (China) to the USA and also in *Cantalupensis* and *Inodorus*, while large- and small-seed types were common in areas from Central Asia to Iran.

Sex expression type, determined by CAPS analysis of *CmACS7*, also differed among melon populations

**Table 3** Number of accessions examined in each population and their characterization based on seed length, sex expression and gene diversity

Population	No. of accessions			Gene diversity
	Total	Small-seed type	Monoecious type	
Cambodia				
East	15	14	15	0.201
North	16	13	16	0.171
Center	5	5	5	0.105
South	19	18	19	0.246
West	7	6	7	0.183
Yunnan (China)	5	5	4	0.084
Thailand	5	2	1	0.328
Myanmar	36	32	34	0.428
Viet Nam				
Dua thom	20	20	20	0.154
Dua dai	1	1	1	-
Dua bo	14	14	0	0.142
Dua gang	13	13	1	0.150
Dua le	8	8	0	0.176
Dua vang	2	2	0	-
Conomon	9	8	1	0.227
Makuwa	11	11	2	0.168
Agrestis	6	6	3	0.105
Xinjiang (China)	24	0	0	0.246
Central Asia	14	7	4	0.351
Pakistan	11	7	4	0.322
Afghanistan	9	3	4	0.252
Iran	10	2	1	0.353
Spain	8	0	0	0.156
USA	5	0	0	0.219
Cantalupensis	10	0	0	0.278
Inodorus	8	0	0	0.240
Total	291	197	142	0.220



(Table 3). All of the Cambodian melon accessions (62/62) proved to be monoecious. The monoecious type is also common in neighboring countries, such as Yunnan (China), Myanmar, and Vietnam (Dua thom). In contrast, andromonoecious type was common in Conomon, Makuwa, Vietnam (except Dua thom), in areas from Xinjiang (China) to the USA, and in *Cantalupensis* and *Inodorus*. Both monoecious and andromonoecious types were frequent in areas from Central Asia to Afghanistan.

#### RAPD and SSR analysis

Ten RAPD markers produced 12 polymorphic bands whose size ranged from 700 to 1520 bp, and the average number of marker bands per primer was 1.2 (Table 2). The average of PIC index was 0.297 and ranged from 0.047 (B32\_700) to 0.452 (B99\_1400). SSR analysis with 7 primer pairs generated 70 bands polymorphic in 291 accessions of melon and the average number of marker bands per primer was 10.0. The PIC index ranged from 0.664 (CMN04-07) to 0.891 (CMBR2) and was almost doubled that of the RAPD markers (Table 2).

Gene diversity (D) in Cambodian melon ranged from 0.105 (center) to 0.246 (south), and was 0.228 in 62 accessions (Table 3). This value was smaller compared with those in neighboring countries such as Vietnam (0.333 in 58 accessions), Myanmar (0.428), and Thailand (0.328), and nearly equivalent to those of Conomon and Makuwa (0.227 and 0.168).

#### Genetic relationship between Cambodian melon and reference accessions

Genetic distance (GD) among 291 accessions ranged from 0.000 (CAn33 vs CA n44 etc.) to 0.947 (P142 vs X8), while the maximum GD was 0.474 (CAs46 vs CAe9 etc.) in Cambodian accessions (data not shown). A dendrogram was constructed based on these GD values, and 291 accessions were grouped into 3 major clusters that were further separated into 24 subclusters (Fig. 2). Cluster I comprised 115 accessions, which included landraces from Cambodia (59/62) and neighboring countries such as Vietnam (Dua thom), Yunnan (China), Thailand, and Myanmar (Table 4). Accessions of Conomon, Makuwa, and Agrestis formed cluster II (68 accessions), together with landraces from Vietnam except

Dua thom. Cluster III (108 accessions) consisted of *Cantalupensis*, *Inodorus*, and accessions from Xinjiang (China) to the USA. Landraces of Myanmar were classified into three clusters in good accordance with the highest D value (0.428).

Structure analysis indicated the presence of 3 main populations in 291 accessions studied (Fig. 2), based on the  $\Delta K$  value, and 61, 101, and 93 accessions were assigned to populations P1, P2, and P3, respectively, using a Q-value threshold of 70% (Table 4). The remaining 36 accessions were regarded as admixture type. The classifications of 3 clusters and 3 populations were highly associated, as 86.1, 88.2, and 85.2% of accessions in clusters I, II, and III were assigned to P1, P2, and P3, respectively. Accessions of admixture type were mostly found in marginal subclusters such as If, Iig, and IIIa. Accordingly, Cambodian accessions belonged to P2 together with those from Vietnam (Dua thom), Yunnan (China), Thailand, and Myanmar, and the genetic relationship detected by cluster analysis was reproduced by structure analysis. A genetic relationship was also visualized on the scatter plot of PCO1 (21.3%) and PCO2 (16.5%) (Fig. 3). Accessions of three populations were distinctly separated and admixture accessions were located among three groups.

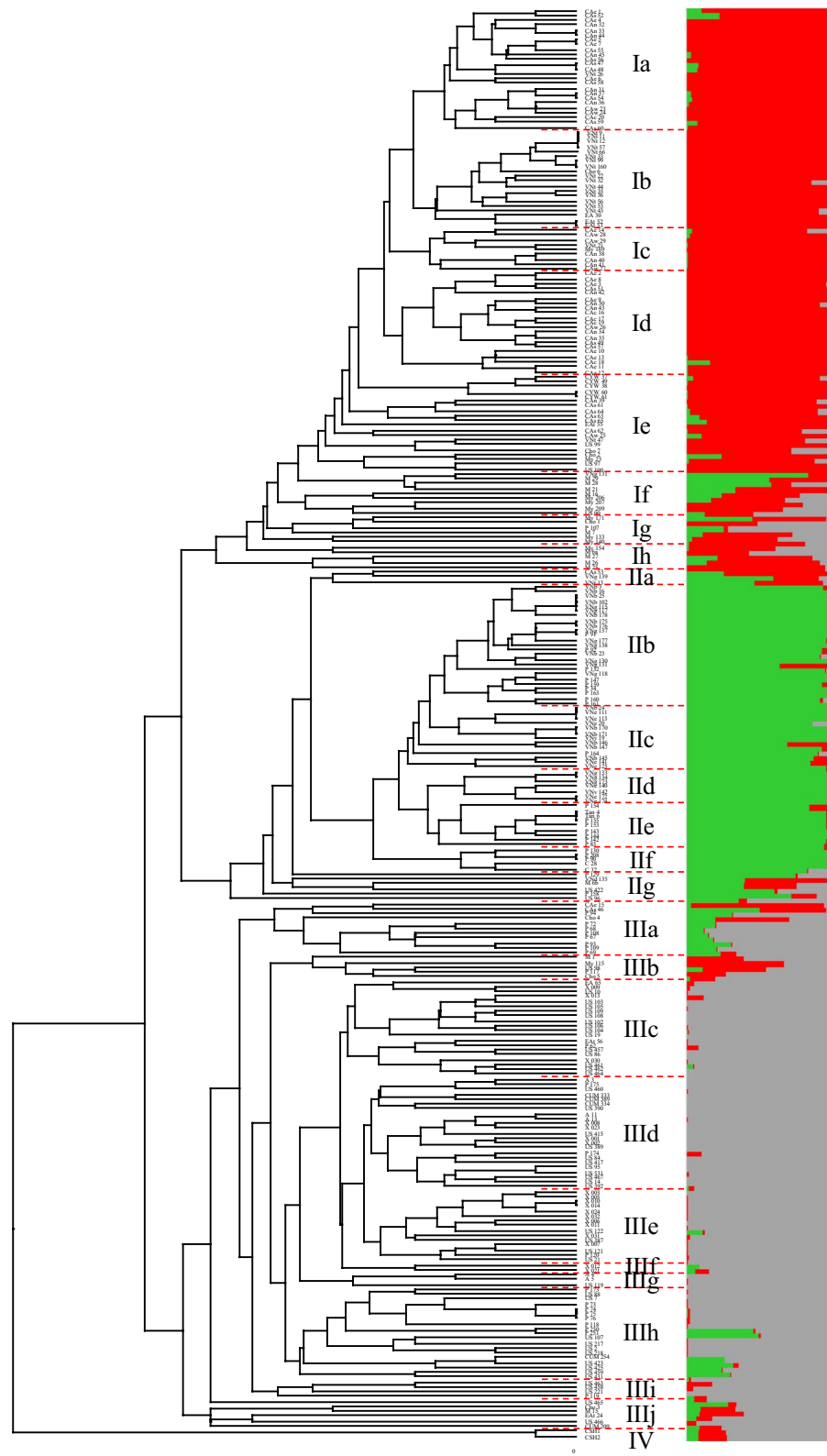
Three clusters were also characterized by seed length and sex expression type (Tables 3 and 4). Most of accessions of clusters I (111/115) and II (65/68) commonly had seeds shorter than 9 mm in length and were classified as small-seed type. In contrast, the sex expression type differed between clusters I and II, and monoecious type and andromonoecious type were common in clusters I (108/115) and II (56/68), respectively. Cambodian melon proved to be small-seed type and monoecious type (56/62), similar to those of Vietnam (Dua thom), Yunnan (China), and Myanmar, thus showing close similarity among these populations. Accessions of cluster III were mostly large-seed type (80/108) and andromonoecious type (87/108).

#### Genetic relationship between 24 melon populations

The genetic distance (GD) between 24 populations calculated from the RAPD and SSR data ranged from 0.018 (Cambodia-North vs Cambodia-South) to 0.984 (Makuwa vs Xinjiang (China) (Table 5). GD among 5 populations of Cambodian melon ranged



**Fig. 2** Genetic relationship between 293 accessions revealed by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on genetic distance, and population structure inferred by STRU CTURE using the admixture model

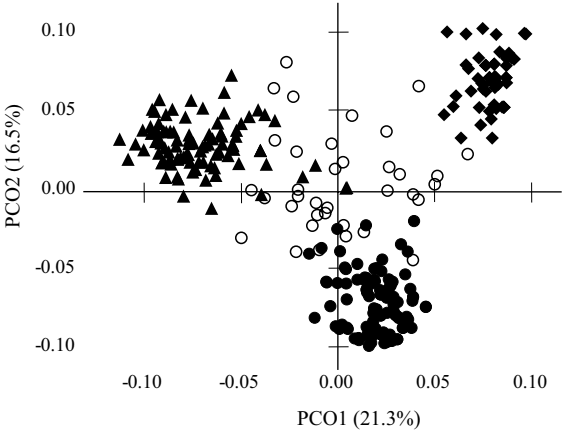


**Table 4** Number of melon accessions classified into 24 subclusters and three populations in Fig. 2

Population	No. of accessions	Cluster No.	Population structure																						
			Ia	Ib	Ic	Id	Ie	If	Ig	Ih	Iia	Iib	Iic	Iid	Iie	IIf	IIIg	IIIh	IIIi	IIIj	IV	P1	P2	P3	Mix
Cambodia	15	5	-	1	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	-	-
	16	7	-	3	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16	-	-
	5	1	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
	19	9	-	-	3	5	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	18	-	1
	7	2	-	3	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-
	5	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
	(China)																								
		5	-	2	-	-	1	-	-	-	-	-	1	-	-	-	-	-	1	-	-	-	3	2	-
	Thailand	36	-	2	1	-	6	7	5	5	-	-	4	1	-	-	-	-	2	-	-	-	13	2	21
	Myanmar																								
	Viet Nam																								
	Dua thom	20	1	16	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19	-	1
	Dua dai	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	Dua bo	14	-	-	-	-	-	-	-	-	-	8	6	-	-	-	-	-	-	-	-	14	-	-	-
	Dua gang	13	-	-	-	-	-	1	-	-	-	8	-	3	-	-	-	-	-	-	-	11	-	-	2
	Dua le	8	-	-	-	-	-	-	-	-	-	-	5	3	-	-	-	-	-	-	-	8	-	-	-
	Dua vang	2	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	2	-	-	-
	Conomon	9	-	-	-	-	-	-	-	-	-	3	-	-	3	1	2	-	-	-	-	9	-	-	-
	Makuwa	11	-	-	-	-	-	-	-	-	-	1	-	-	6	4	-	-	-	-	-	11	-	-	-
	Agrestis	6	-	-	-	-	-	-	-	-	-	5	1	-	-	-	-	-	-	-	-	6	-	-	-
Xinjiang (China)	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	7	10	2	2	-	24	-	
Central Asia	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	2	-	1	3	-	12	2	
Pakistan	11	-	-	-	-	-	-	-	-	-	-	-	4	2	-	-	1	2	2	-	-	-	9	2	
Afghanistan	9	-	-	-	-	-	-	-	-	-	-	-	2	4	2	-	1	-	-	-	-	-	9	-	
Iran	10	-	-	-	-	-	1	-	-	-	-	-	1	3	-	-	4	-	-	-	-	-	7	3	
Spain	8	-	-	-	-	-	-	-	-	-	-	-	7	-	-	-	1	-	-	-	-	-	8	-	
USA	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	1	-	-	-	-	5	-	
Catalunpensis	10	-	-	-	-	-	-	1	-	-	-	-	8	1	-	-	-	-	-	-	-	-	7	3	
Inodorus	8	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	5	1	-	-	-	8	-	

**Table 4** (continued)

Population	No. of accessions	Cluster No.	Population structure																												
			Ia	Ib	Ic	Id	Ie	If	Ig	Ih	Iia	Iib	Iic	Iid	Iie	Iif	Iig	Iih	Iii	Iij	IV	P1	P2	P3	Mix						
Hard-wickii	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2	-						
Total	293	25	20	9	21	20	9	6	5	3	25	13	7	9	5	6	11	5	20	23	15	2	3	19	4	6	2	61	101	95	36
Association with population structure																															
P1	61	-	-	-	-	-	-	1	-	-	0	24	13	7	9	5	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2	101	25	20	9	21	20	1	1	2	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P3	95	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	5	2	20	23	15	2	3	15	4	3	2	-	-	-
Mix	36	-	-	-	-	-	-	7	4	3	2	1	-	-	-	-	4	5	3	-	-	-	-	-	4	-	3	-	-	-	-



**Fig. 3** Distribution of 293 accessions on the first two principal coordinates. Accessions are indicated with symbols unique to each population; P1 (◆), P2 (●), P3 (▲), and Admixture (○)

from 0.018 to 0.097, and was 0.062 on average. These values were nearly equivalent to those among 3 Vietnamese populations of Dua bo, Dua gang and Dua le (0.035–0.082), among Conomon and Makuwa (0.047) and among 5 populations from Xinjiang (China) to Iran (0.044–0.112), indicating that Cambodian melons were not genetically differentiated among geographical populations.

The genetic relationship between 24 populations was visualized by cluster analysis (Fig. 4) and by PCO analysis (Fig. 5). In accordance with the results of accession-based analysis, 24 populations were separated into 3 groups and Cambodian populations related closely to those in neighboring countries.

**Discussion**

The distribution of Conomon and Makuwa is limited to the eastern part of Asia, including China, Vietnam, Korea and Japan, and thus Conomon and Makuwa were considered as being introduced from the West. Previous research using isozyme and molecular markers have suggested that Conomon and Makuwa originated from small-seed melons with Ia type cytoplasm in areas lying somewhere between India and China (Akashi et al. 2002; Tanaka et al. 2007; Serres-Giardi and Dogimont 2012), and Tanaka et al. (2007) confirmed the presence of landraces, which were clustered together with Conomon and Makuwa, in North-east India. These results highlight the importance

**Table 5** Genetic distance between 24 populations based on the frequency of 10 random amplified polymorphic DNA (RAPD) and seven simple sequence repeat (SSR) markers

Popula- tion	Cam- bodia- East	Cam- bodia- North	Cam- bodia- Center	Cam- bodia- South	Cam- bodia- West	Yunnan (China)	Thai- land	Myan- mar	Viet- nam- thom	Viet- nam- bo	Viet- nam- gang	Viet- nam- le	Cono- mon	Makuwa	Agres- tis	Xin- jiang (China)	Central Asia	Paki- stan	Afgha- nistan	Iran	Spain	USA	Can- talu- pensis
Cambo- dia- North	0.045																						
Cambo- dia- Center	0.055	0.097																					
Cambo- dia- South	0.045	0.018	0.089																				
Cambo- dia- West	0.071	0.064	0.078	0.057																			
Yunnan (China)	0.243	0.203	0.311	0.181	0.250																		
Thailand	0.165	0.186	0.246	0.166	0.206	0.268																	
Myanmar	0.137	0.123	0.226	0.110	0.136	0.200	0.114																
Vietnam- Dua thom	0.138	0.103	0.203	0.123	0.146	0.141	0.194	0.121															
Vietnam- Dua bo	0.431	0.425	0.570	0.385	0.478	0.511	0.445	0.353	0.518														
Vietnam- Dua gang	0.373	0.397	0.493	0.359	0.429	0.517	0.455	0.337	0.482	0.035													
Vietnam- Dua le	0.541	0.565	0.676	0.524	0.611	0.675	0.541	0.472	0.662	0.077	0.082												
Conomon	0.448	0.505	0.553	0.427	0.528	0.524	0.454	0.386	0.575	0.086	0.060	0.089											
Makuwa	0.568	0.618	0.695	0.553	0.651	0.676	0.554	0.502	0.721	0.115	0.101	0.059	0.047										
Agrestis	0.608	0.567	0.818	0.499	0.623	0.615	0.627	0.479	0.660	0.100	0.093	0.118	0.119	0.117									
Xinjiang (China)	0.529	0.560	0.669	0.523	0.577	0.553	0.322	0.308	0.563	0.841	0.825	0.942	0.868	0.984	0.936								
Central Asia	0.407	0.460	0.510	0.415	0.455	0.536	0.271	0.250	0.501	0.662	0.620	0.697	0.654	0.731	0.753	0.060							
Pakistan	0.485	0.533	0.588	0.488	0.496	0.676	0.267	0.302	0.624	0.677	0.631	0.699	0.665	0.715	0.760	0.109	0.099						
Afghani- stan	0.472	0.514	0.595	0.478	0.523	0.636	0.291	0.313	0.602	0.723	0.720	0.751	0.775	0.844	0.867	0.070	0.044	0.089					
Iran	0.420	0.445	0.549	0.408	0.452	0.618	0.254	0.249	0.549	0.557	0.545	0.522	0.585	0.586	0.627	0.112	0.048	0.089	0.058				
Spain	0.495	0.533	0.652	0.494	0.546	0.613	0.302	0.312	0.579	0.717	0.690	0.804	0.716	0.812	0.855	0.137	0.144	0.177	0.152	0.159			

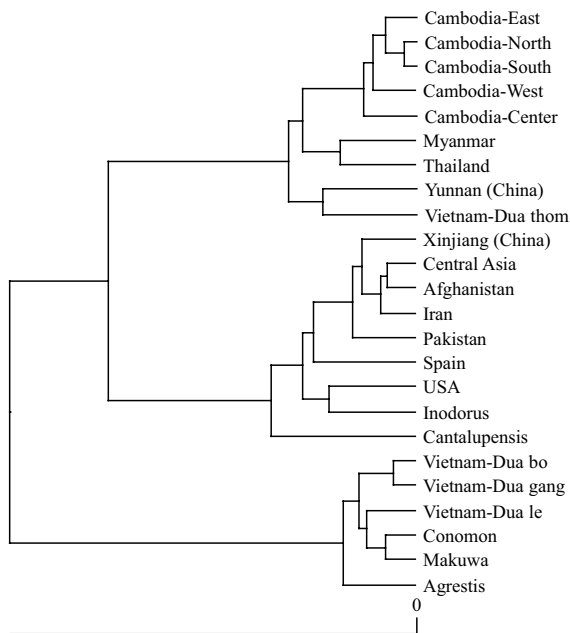
Table 5 (continued)

Popula- tion	Cam- bodia- East	Cam- bodia- North	Cam- bodia- Center	Cam- bodia- South	Cam- bodia- West	Yunnan (China)	Thai- land	Myan- mar	Viet- nam- thom	Viet- nam- bo	Viet- nam- gang	Viet- nam- Dua le	Cono- mon	Makuwa	Agres- tis	Xin- jiang (China)	Central Asia	Paki- stan	Afgha- nistan	Iran	Spain	USA	Can- talu- pensis
USA	0.504	0.492	0.651	0.440	0.523	0.621	0.296	0.325	0.619	0.665	0.647	0.670	0.703	0.791	0.824	0.215	0.169	0.230	0.184	0.138	0.200		
Cantalu- pensis	0.384	0.453	0.479	0.417	0.457	0.629	0.276	0.262	0.547	0.525	0.455	0.540	0.467	0.522	0.563	0.268	0.150	0.209	0.206	0.164	0.292	0.303	
Inodorus	0.362	0.373	0.462	0.324	0.388	0.533	0.222	0.265	0.477	0.577	0.581	0.651	0.625	0.710	0.762	0.185	0.108	0.164	0.141	0.115	0.209	0.130	0.161

of genetic research of melon landraces in Southeast Asia. However, few accessions are available in the public genebank worldwide. Therefore, we collected melon germplasm from Southeast Asian countries and analyzed genetic structure in Myanmar (Yi et al. 2009) and Vietnam (Nhi et al. 2010; Duong et al. 2021). This research indicated genetic differentiation in melon landraces in Southeast Asian countries, and highlighted the importance of genetic research using landraces from Cambodia and Laos.

The joint expedition team of NARO (Japan) and CARDI (Cambodian Agricultural Research and Development Institute, Cambodia) conducted melon germplasm collection in the western (2014), eastern (2015), central and northern (2016) and southern (2017) regions (2017) of Cambodia during 2014–2017 (Fig. 1). The geographical distribution, horticultural characteristics, and usage of melon landraces in Cambodia were first fully described by their reports (Matsunaga et al. 2015; Tanaka et al. 2016, 2017, 2019). According to their reports, non-netted melon with low sugar content was commonly grown in every part of Cambodia and utilized mature fruit as a desert, and immature fruit as vegetable or pickles. Immature and mature fruits of Conomon are mainly used for making pickles in Japan, suggesting the similarity between Cambodian melons and Conomon. To discuss the origin of Conomon and Makuwa, using part of this collection, we uncovered the genetic diversity of Cambodian melons in the present study, and compared it with those in neighboring countries.

Genetic diversity of cultivated melons is rich in India (Akashi et al. 2002; McCreight 2004) and this country was once considered the secondary center of diversity. However, based mainly on the discovery of wild perennial melon (*C. trigonus*), India is now considered as one of the domestication centers of melons along with Africa (John et al. 2013; Endl et al. 2018). Moreover, large genetic variations in Indian melons have been reported in various traits (Dhillon et al. 2007, 2009; Fergany et al. 2011; Roy et al. 2012). Seed size variation was also studied, and both large- and small-seed types are commonly distributed in India, with the latter being rather frequent in North-eastern India (Akashi et al. 2002; Tanaka et al. 2007). In contrast, small-seed type is frequent in Myanmar (36/41, Yi et al. 2009), Cambodia (58/58, Table 3), and Vietnam (61/61, Table 3), indicating an increasing trend of small-seed type from India to Vietnam.



**Fig. 4** Genetic relationship between 24 populations of melon, revealed by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on genetic distance

Genetic diversity also showed a similar geographical cline. *D* was larger in India compared to Myanmar (Yi et al. 2009), while in the present study (without Indian accessions), it was highest in Myanmar (0.428, Table 3) followed by Vietnam (0.333) and Cambodia (0.228). Although *D* was rather high in Thailand (0.328), the proportion of small-seed melons was exceptionally low (40%), indicating a necessity of diversity study using more landraces.

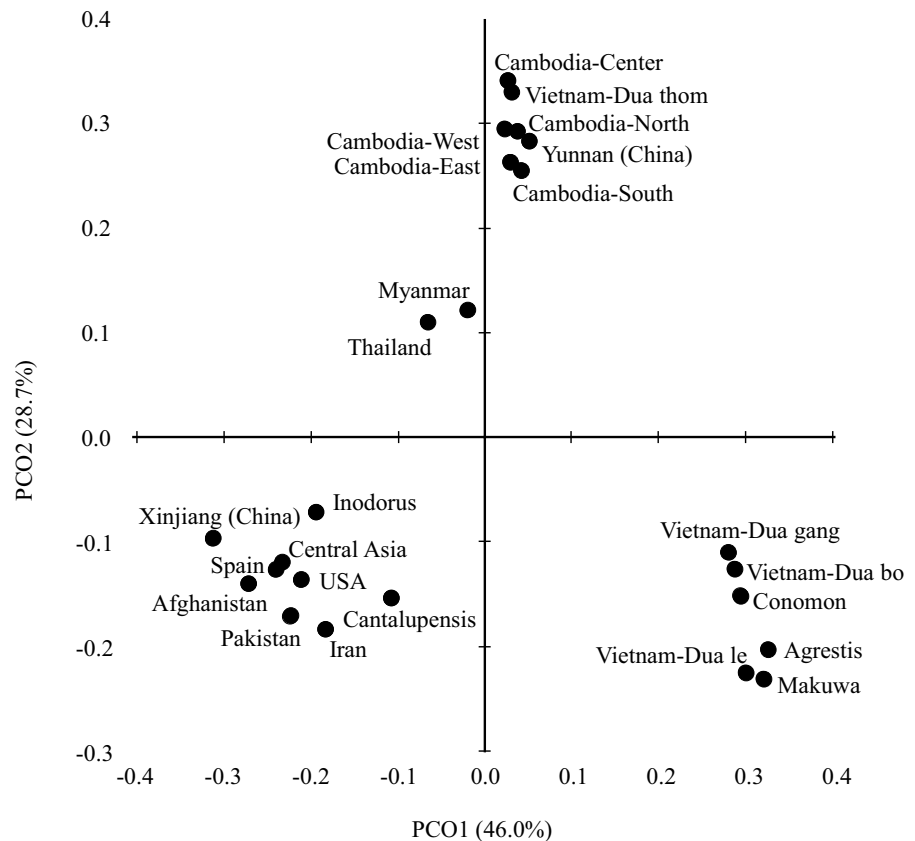
In areas from South to Southeast Asia, Myanmar landraces are closely related to Indian landraces (Tanaka et al. 2007; Yi et al. 2009), and as well as those of Thailand, Cambodia, Yunnan (China), and Vietnam (Dua thom) (Table 4, Fig. 4). These results indicated the introduction of non-netted melons from India to Southeast Asia and the decrease of genetic variation by natural and artificial selection. In this area, various ethnic groups of Tibeto-Burman or Austroasiatic language families live intricately across political borders (Clarke 2001). For example, the “Karen” tribe lives in Northeastern India, Myanmar, and Thailand, and the “Hmong” tribe lives in Laos, Thailand, and Vietnam. Local people definitely played a crucial role in the

trade of goods (Misra 2005; Shibayama 2019) and the spread of melon from India to the East.

Southeast Asian melon landraces comprised two genetic groups, as clearly shown in Table 4 and Figs. 3, 4 and 5. The first group consisted of landraces of cluster I and population P2, which are commonly grown in Myanmar, Thailand, Cambodia, Yunnan (China), and mountain area of Vietnam (Dua thom). The second group consisted of landraces of cluster II and population P1, including Conomon and Makuwa, *Agrestis* (weedy melon) collected in Japan and Korea, and landraces grown in the plains of Vietnam. Landraces of both groups show similarities in several traits, such as smooth skin without net and small seeds shorter than 9 mm, and so should be classified as *ssp. agrestis*, according to the former systematics by Pitrat (2013). In addition, both groups belong to the same maternal lineage (Ia), as revealed by the analysis of chloroplast genome markers (Tanaka et al. 2013; Tanaka et al. in preparation). However, they also differed in several traits, reflecting their genetic differentiation. The key trait is sex expression type, and the first and second groups showed monoecy and andromonoecy, respectively (Table 3). Flesh sweetness also differs among groups, but the difference is not simple and only Makuwa of the second group has sweet flesh.

It was therefore concluded that melon landraces predominantly grown in most parts of Southeast Asia, including Cambodia, were of the first group and those of the second group were limited to the plains of Vietnam. In Vietnam, the second group is grown in the plains by “Kinh” tribe originally migrated from southern part of China, whereas the first group is grown in mountain areas by ethnic groups “Hmong” and “Thai” in northern part and by ethnic groups “Jarai” and “Hmong” in southern part (Duong et al. 2021). Therefore, the allopatric distribution of ethnic groups was considered as the primary reason for the presence of two genetic groups in Vietnam, though the possibility of severe selection due to pests, insects and so on could not be ruled out. In case of Cambodia, it shares the border with southern part of Vietnam where “Khmer” tribe lives as ethnic group, and thus the introduction of melon landraces across the border was reasonably hypothesized. However, landraces of different genetic groups were grown on both sides of the border, and Conomon and Makuwa, or their ancestral type were not found in Cambodia.

**Fig. 5** Distribution of 24 populations of melon on the first two principal coordinates



Therefore, Cambodian melon is not directly related with the establishment of Conomon and Makuwa, supporting the introduction of Conomon and Makuwa from southern part of China to Vietnam as indicated by Duong et al. (2021). To further confirm this conclusion, landraces from Thailand, Laos, and Yunnan (China) should be studied in detail.

**Author contributions** All authors contributed to the study conception and design. Field research and the collection of landraces were prepared and performed by Katsunori Tanaka, Sakhan Sophany, Ouch Sreynech, Gentaro Shigita, Yon Sophea, Ouk Makara, Norihiko Tomooka, and Kenji Kato. DNA experiments were performed by Pervin Mst Naznin, Od-irichi Nnennaya Imoh, and Katsunori Tanaka. The first draft of the manuscript was written by Pervin Mst Naznin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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#### Declarations

**Conflict of interest** The authors have not disclosed any competing interests.

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