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Phylogenetic relationships among cultivated types of *Brassica rapa* L. em. Metzg. as revealed by AFLP analysis

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Abstract

The cultivated types of *Brassica rapa* L. em. Metzg. consist of morphologically distinct subspecies such as turnip, turnip rape, Chinese cabbage, pak choi and pot herb mustard which are classified as ssp. *rapa*, ssp. *oleifera*, ssp. *pekinensis*, ssp. *chinensis* and ssp. *nipposinica* (syn. ssp. *japonica*), respectively. We attempted to elucidate the phylogenetic relationships among the cultivated types of *B. rapa*. Thirty-two accessions from the Eurasian Continent were analyzed using AFLP markers with a cultivar of *B. oleracea* as an outgroup. In total, 455 bands were detected in the ingroup and 392 (86.6%) were polymorphic. The Neighbor-Joining tree based on the AFLP markers indicated that the accessions of *B. rapa* were congregated into two groups according to geographic origin. One group consisted of ssp. *rapa* and ssp. *oleifera* of Europe and Central Asia and the other included all the subspecies of East Asia. Our results suggest that cultivars from East Asia were probably derived from a primitive cultivated type, which originated in Europe or in Central Asia and migrated to East Asia. This primitive cultivated type was probably a common ancestor of ssp. *rapa* and ssp. *oleifera*. The Neighbor-Joining tree also shows that leafy vegetables in East Asia such as ssp. *pekinensis*, ssp. *chinensis* and ssp. *nipposinica* were differentiated several times from the distinct cultivars of ssp. *oleifera* in East Asia.

Introduction

Brassica rapa L. em. Metzg. (syn. *B. campestris* L. see Oost et al. 1987) includes wild, weedy and cultivated types. The wild type is distributed widely from Europe to Central Asia (De Candolle 1886; Tsunoda 1980), and the cultivated type, which originated in this area, now prevails throughout the world as several kinds of vegetables (reviewed by Prakash and Hinata 1980). The cultivated forms of *B. rapa* consists of morphologically distinct infraspecific types, which are distinguished by the morphology of their edible or useful parts, such as swollen roots or fleshy leaves for vegetable use, or plentiful seed production for seed oil use. Hybrids between these infraspesific types are fertile, so that each type was not classified as a distinct species, but was classified as a subspecies. They are *B. rapa* ssp. *rapa* (turnip, which has edible swollen roots), ssp. *oleifera* (DC.) Metzg. (turnip rape, or Chinese colza, used for seed-extracted oil), ssp. *pekinensis* (Lour.) Hanelt (Chinese cabbage, cabbage like vegetable formed by tightly overlapping pale green leaves), ssp. *chinensis* (L.) Hanelt (pak choi or Chinese mustard, leafy vegetable with large, thick leaves, broad thick white petioles) and ssp. *nip-*

posinica (Bailey) Hanelt (syn. ssp. *japonica* (Bailey) Olsson; pot herb mustard, leafy vegetable forming a fairly large stump with many narrow thin leaves) (Olsson 1954; Gladis and Hammer 1992).

The cultivated type of *B. rapa* was classified into two major groups based on morphology (Sinskaia 1928; Prakash and Hinata 1980) and restriction fragment length polymorphism (RFLP) markers (Song et al. 1988; Crouch et al. 1995). One group consists of ssp. *rapa* and ssp. *oleifera* in Europe and another is the group of leafy vegetables, such as ssp. *pekinensis*, ssp. *chinensis* and ssp. *nipposinica* in East Asia. Song et al. (1988, 1990) analyzed them by RFLPs and concluded that the two groups originated from distinct wild populations from Europe and China, respectively. However, they did not include ssp. *rapa* and ssp. *oleifera* from East Asia in their studies. Furthermore, it has been well known that natural populations of *B. rapa* do not exist in East Asia, (Tsunoda 1980). Hence the origin of the leafy vegetables and of the East Asian ssp. *rapa* and ssp. *oleifera* still remains unknown.

The amplified fragment length polymorphism (AFLP) technique, developed by Vos et al. (1995), is more effective, more cost efficient and more reproducible method in revealing polymorphisms than are RFLPs (Ajimone et al. 1998 Bohn et al. 1999). This method has been successfully applied to many phylogenetic studies on plants (e.g. barley, Badr et al. 2000) and on animals (e.g. cichlid fish, Albertson et al. 1999). In this study, we used AFLP markers to elucidate the phylogenetic relationships among 32 accessions of five subspecies of *B. rapa* sampled from around the Eurasian Continent, including the ssp. *rapa* and ssp. *oleifera* from East Asia. The probable domestication processes and the diffusion routes of the cultivated types of *B. rapa* will be discussed based on their phylogenetic relationships.

Materials and methods

Plant materials

Thirty-two accessions of *B. rapa* were sampled from around the Eurasian Continent and included 17 accessions of *B. rapa* ssp. *rapa*, 3 of ssp. *oleifera*, 4 of ssp. *pekinensis*, 3 of ssp. *chinensis*, 2 of ssp. *nipposinica* and 3 accessions of which the subspecies name were not labeled (Table 1). Since ssp. *rapa* was known to be the most widely distributed ancient type of cultivated form of *B. rapa* and possess high morphological variations (Sinskaja 1928), we sampled many accessions of ssp. *rapa* around the Eurasian Continent. Sixteen accessions of the 32 were obtained from the IPK, Gatersleben, Germany, and 7 from the MAFF Gene bank, Tsukuba, Japan. All samples obtained from the IPK and the MAFF were landraces. Also, nine commercial cultivars collected by one of the authers (O. O.) were also used in this study. The cabbage cultivar, 'Ajiboshi' (*B. oleracea*) was used as an outgroup.

For a technical reason, we had to limit the number of accessions used in AFLP analysis in order to precisely infer the phylogenetic relationships of *B. rapa*. More accessions we used, more difficult we are to judge by eye whether two allelic bands were the same or not.

All the accessions were grown from the seed sample in a greenhouse or field at Plant Germplasm Institute of Kyoto University, Kyoto, Japan in order to permit observations on morphological characters. All the accessions, except for three, were labeled with the subspecies name. For each accession, we carefully observed and checked the morphological traits of the labeled subspecies name according to Olsson (1954, see Introduction).

We also inferred the subspecies name of three un-labeled accessions. The accession r_{18} of Iraq from IPK had a swollen root; hence it may belong to ssp. *rapa*. The accession of China from IPK did not have any swollen root, and had relatively thick leaves; hence it may be either ssp. *chinensis* or ssp. *oleifera*. This accession was designated as x1. The commercial cultivar of China bolted earlier than other cultivars and did not have a swollen root. This suggests that this cultivar may be classified as ssp. *oleifera* and was designated as o_1.

DNA extraction and AFLP analysis

The total DNA was extracted from young leaves of a representative individual for each accession by the CTAB method according to Escaravage et al. (1998).

Table 1. The list of Brassica accessions used in this study.

ID symbol	Species	Subspecies	Name	Country of Origin	Source	Accession No.
r_1	*B. rapa*	*rapa*	Italiaanse Witte Roodkop	Italy	IPK	BRA1115
r_2			Opava	Czech	IPK	BRA1023
r_3			Hammenhoegs Bortfelder	Sweden	IPK	BRA329
r_4			Hilversumse	Netherlands	IPK	BRA1013
r_5			Horpacsi Lila	Hungary	IPK	BRA1017
r_6			Teltower Ruebchen	Germany	IPK	BRA1700
r_7			Salgam	Tadzhikistan	IPK	K8266
r_8			Namanganskaja	Russia	IPK	BRA1719
r_9			unknown	Georgia	IPK	BRA 1715
r_{10}*			Blanc Plat Hatif	France		
r_{11}*			Kranjska Okrugla	Slovenia		
r_{12}*			Purple turnip	Greece		
r_{13}*			Purple top white globe	India		
r_{14}*			Turnip Purple Top	Nepal		
r_{15}*			Kang Hwa	South Korea		
r_{16}			Kanamachi kokabu	Japan	MAFF	26852
r_{17}			Tennoji kabu	Japan	MAFF	26870
r_{18}			unknown	Iraq	IPK	K7170
p_1*		*pekinensis*	Nagaoka F1	Slovenia		
p_2			Peking Pai Kou	China	IPK	BRA124
p_3			Wong Bok	North Korea	IPK	BRA473
p_4			Kurihara santo	Japan	MAFF	26711
o_1*		*oleifera*	Kuan bang ging cai	China		
o_2			Hisagona	China	IPK	K8511
o_3			Torkel	Sweden	IPK	K7968
o_4			Fukidachi	Japan	MAFF	25936
c_1		*chinensis*	Chinese Kwongjin	China	IPK	BRA462
c_2			Known You Spoon Pak 78A	Taiwan	IPK	BRA1634
c_3			Sendai yukina	Japan	MAFF	26062
n_1		*nipposinica*	Shirakuki sensuji kyomizuna	Japan	MAFF	43287
n_2			Chudoji mibuna	Japan	MAFF	26100
x_1		unknown	unknown	China	IPK	K9708
Bo*	*B. oleracea*		Ajiboshi			

*Commercial cultivars.

The AFLP reactions were carried out according to the manufacturer's protocol (AFLP Analysis Kit; Invitrogen corp., Carlsbad, CA, U.S.A.). One and three selective nucleotides were used at pre- and selective amplification, respectively. Thirteen primer combinations were used in selective amplifications (Table 2). DNA fragments were resolved by electrophoresis on a 5% denaturing polyacrylamide gel and they were then visualized with a silver staining kit (Promega, Madison, Wis., U.S.A.).

Data analysis

Only the AFLP bands, which could be clearly distinguished by eye, were manually scored as present (1) or absent (0) and each band was regarded as a locus. The genetic distance between all pairs of accessions was estimated on the basis of the Jaccard coefficient (Jaccard 1908). To infer the phylogenetic relationships among accessions, the matrix of genetic distance was analyzed using the Neighbor-Joining method (Saitou and Nei 1987). The reliability of each branch in the NJ tree was tested by bootstrap analysis (Felsenstein 1985) with 1000-replicated data sets. These procedures were conducted using TREECON version 1.3 (Van de Peer and De Wachter 1994).

In this study, we used only one individual for each accession. This may lead to an error by chance in inferring phylogenetic relationships. However, our previous AFLP analysis on Japanese landraces of *B. rapa* suggested that using either one or five individuals per accessions made no difference in phylogeny of *B. rapa* (Takuno, unpublished results).

Table 2. No. of detected bands for each primer pair*.

Primers		No. of bands	No. of polymorphic bands	Percentage of polymorphic bands
Eco RI	*Mse* I			
AAC	CAA	35	28	80.00
AAG	CAA	39	32	82.05
AAG	CAC	43	38	88.37
ACC	CAG	26	24	92.31
AGG	CAG	35	29	82.86
ACT	CAT	50	43	86.00
AAC	CTA	32	27	84.38
AGG	CTA	25	22	88.00
ACT	CTC	31	30	96.77
AAG	CTC	41	37	90.24
ACA	CTT	47	39	82.98
ACT	CTT	25	24	96.00
ATT	CGT	26	21	80.77
Average		35	30.3	86.59
All		455	394	86.59

* Only the bands detected in ingroup samples were counted.

Results

The 13 AFLP primer combinations generated a total of 571 bands. The molecular weight of the bands ranged from approximately 100 to 600 bp. When the bands specific to the outgroup, *B. oleracea*, were excluded, 455 bands were detected, of which 394 (86.6%) were polymorphic, with an average of 30.3 polymorphic bands per primer combination (Table 2). The number of bands scored per accession ranged from 179 in cultivar p_2 to 221 in cultivar r_{10} with an average of 197. No band was found to be subspecies-specific.

In the Neighbor-Joining tree in Figure 1, only bootstrap values of larger than 50% are shown. The inferred tree indicated that the accessions of *B. rapa* fell into two groups according to their geographic origin. One group consisted of the accessions from Europe to India and the other was of the cultivars from East Asia ranging from Nepal to Japan. They were designated as group W (Western) and E (Eastern), respectively. High bootstrap values of 98 and 80% supported the clades of W and E, respectively. The East Asia group included a Slovenian commercial cultivar p_1. However, this cultivar was known to have been introduced from Japan, hence the result was not surprising. The accessions of ssp. *rapa* and ssp. *oleifera* from East Asia fell into the group E. With the exception of ssp. *nipposinica*, none of the subspecies was monophyletic in the tree.

The group W included 14 accessions of *B. rapa* ssp. *rapa* and 1 of ssp. *oleifera*. The group E was composed of all five subspecies, that is, 4 accessions of ssp. *rapa*, 3 of ssp. *oleifera*, 3 of ssp. *chinensis*, 4 of ssp. *pekinensis*, 2 of ssp. *nipposinica* and 1 of unknown taxa (x_1). Accessions in the group E were further subdivided into 3 subgroups, designated as A, B and C in Figure 1. These subgroups were supported by high bootstrap values of 76, 86 and 100%, respectively. The subgroup A consisted of 6 accessions from Japan, 1 from China, 1 from South Korea and 1 from Taiwan. In subgroup B, 5 accessions were leafy vegetables from China, Japan and Korea, and 1 was ssp. *oleifera* from China. The subgroup C included ssp. *rapa* of Nepal and ssp. *oleifera* of China.

Discussion

Previous studies, using morphology and RFLP analysis (Sinskaja 1928; Prakash and Hinata 1980; Song et al. 1988, 1990; Crouch et al. 1995) indicated that ssp. *rapa* and ssp. *oleifera* were distantly related to the leafy vegetables. They also suggested that the ssp. *rapa* -ssp. *oleifera* complex and the leafy vegetables were differentiated from distinct natural populations of wild ssp. *rapa*. However, our result did not agree with this conclusion as ssp. *rapa* and ssp. *oleifera* from East Asia were found to be closely related to the leafy vegetables.

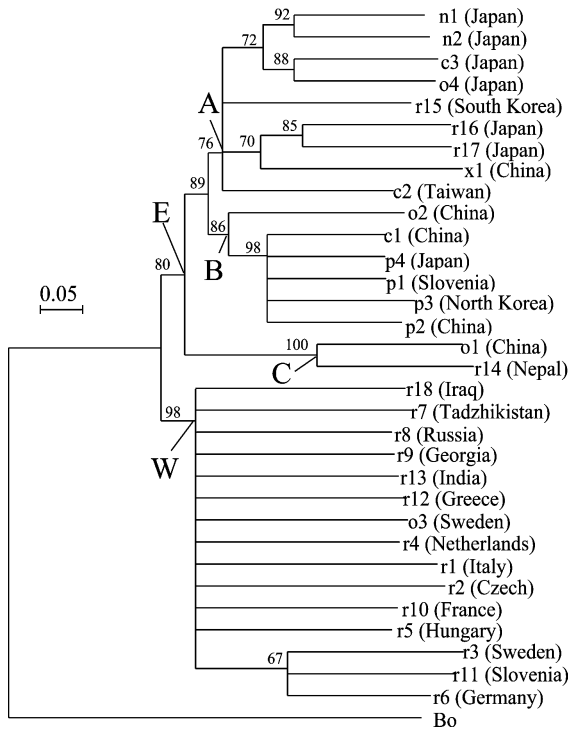

Figure 1. The phylogenetic tree among cultivated types of *Brassica rapa*. For the ID symbol, see Table 1. The numbers above nodes indicate bootstrap values (only the values of larger than 50% are shown). The scale bar represents genetic distance.

The primitive cultivated type of *B. rapa* originated in Europe or Central Asia (Prakash and Hinata 1980). Nishi (1980) considered that primitive cultivated *B. rapa* migrated to East Asia as an agricultural crop and the leafy vegetables subsequently differentiated from this primitive *B. rapa* in China. Our results in Figure 1 are consistent with this hypothesis. Furthermore, the phylogenetic relationships in Figure 1 suggest that a primitive type of *B. rapa* might be the common ancestor of ssp. *rapa* and ssp. *oleifera*, and later differentiated into the two groups. One that spread over the area from Europe to India and the other that migrated to East Asia and dispersed in this area. In the area from Europe to India, various types of ssp. *rapa* (and of ssp. *oleifera*) diverged from the primitive type through geographic isolation and intensive selection by man, whereas in East Asia, first the ssp. *chinensis*, pak choi (BC 5C) then the ssp. *pekinensis*, Chinese cabbage (11C) differentiated from ssp. *rapa* or from ssp. *oleifera* (Aoba 2000).

The existence of the primitive cultivated *B. rapa* at the early stage of domestication argued above is purely an assumption. Alternatively, we may consider that turnip, ssp. *rapa* might was the primitive type of cultivated *B. rapa*, which was originated in Central Asia or in Europe and diffused both to East Asia and to Europe and India. This idea is associated with Sinskaja (1928)'s observations that turnips were classified into seven groups based on regional and morphological variations and the Afghanistan type of Central Asia was an ancestral form of cultivated *B. rapa*. In this case, more extensive surveys in Central Asia (or in Europe) may allow us to find primitive cultivated types of ssp. *rapa*, which are situated at the ancestral node of phylogenetic tree. If we find it, our hypothesis has a solid experimental basis.

The phylogenetic tree, which was developed, (Figure 1) indicates that group E differentiated into three subgroups. The subgroup C contains 1 accession of ssp. *rapa* (r_{18}) from Nepal and 1 of ssp. *oleifera* (o_1) from China. The turnip cultivar, similar to that of Nepal, is extensively cultivated in Tibet. The subgroup C is likely to be the oldest type of the group E since it is most closely situated to the ancestral node. In East Asia, both subgroups A and B contained leafy vegetables. The

subgroup A included both ssp. *rapa* and ssp. *oleifera*, whereas subgroup B only included ssp. *oleifera*. This suggested that leafy vegetables were independently derived from distinct cultivars of ssp. *oleifera* at least twice; and probably many times from both ssp. *rapa* and ssp. *oleifera*.

In addition we can speculate more on their domestication. Since Chinese cabbage, ssp. *pekinensis* developed around the 11-th century in China, and was introduced into Japan only recently (19-th century) (Aoba 2000), the cultivars in subgroup B can be considered to be leafy vegetables (ssp. *chinensis* and ssp. *pekinensis*) which developed in China and their ancestral cultivars, ssp. *rapa* and ssp. *oleifera* of China. Furthermore, the subgroup A consisted of Japanese cultivars, including the new leafy vegetable ssp. *nipposinica*. We can consider that subgroup A consists of Chinese cultivars of ssp. *rapa*, ssp. *oleifera*, and ssp. *chinensis* introduced into Japan, and the newly developed ssp. *nipposinica* in Japan.

Group W consisted of ssp. *rapa* and ssp. *oleifera* cultivars of Europe and Central Asia. It did not include the leafy vegetables ssp. *chinensis*, ssp. *pekinensis* and ssp. *nipposinica*. Extensive development of the vegetables from *B. oleracea*, kale, cabbage, cauliflower and others, in Europe or in Central Asia, instead of the development of leafy vegetable from *B. rapa*, may explain the reason why the leafy vegetables have not developed outside East Asia.

The phylogeny among the accessions of the group W was unclear, but the phylogeny of the group E was relatively well resolved (Figure 1). This implies that the accessions of the group W may considerably rapidly diverge predating allele fixation in each accession, which causes the disruption of a tree topology (Moran and Kornfield 1993). To resolve the phylogeny among the accessions of the group W, we are required to use more rapidly mutated markers like SSRs.

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References

Ajimone M.P., Castigliono P., Fusari F., Kuiper M. and Motto M. 1998. Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. Theor. Appl. Genet. 96: 219–227.

Albertson R.C., Markert J.A., Danley P.D. and Kocher T.D. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. Proc. Natl. Acad. Sci. USA 96: 5107–5110.

Aoba T. 2000. Japanese Vegetables (in Japanese). Yasaka Press, Tokyo, pp. 150–163

Badr A., Müller K., Schäfer-Pregl R., El Rabey H., Effgen S., Ibrahim H.H., Pozzi C., Rohde W. and Salamini F. 2000. On the origin and domestication history of Barley (*Hordeum vulgare*). Mol. Biol. Evol. 17: 499–510.

Bohn M., Utz F.H. and Melchinger A.E. 1999. Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs, and SSRs and their use for predicting progeny variance. Crop Sci. 39: 228–237.

Crouch J.H., Lewis B.G., Lydiate D.J. and Mithen R. 1995. Genetic diversity of wild, weedy and cultivated forms of *Brassica rapa*. Heredity 74: 491–496.

De Candolle A. 1886. Origin of Cultivated Plants. Hafner Publ. Co., New York, 1964 reprint.

Escaravage N., Questiau S., Pornon A., Doche B. and Taberlet P. 1998. Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. Mol. Ecol. 7: 975–982.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Gladis T.H. and Hammer K. 1992. Die Gaterslebener *Brassica*-Kollektion – *Brassica juncea*, *B. napus*, *B. nigra* und *B. rapa* (in German). Feddes Repertorium 103: 7–8, 469–507.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaudoise. Sci. Nat. 44: 223–270.

Moran P. and Kornfield I. 1993. Retention of an ancestral polymorphism in the Mbuna species flock (Teleostei: Cichlidae) of Lake Malawi. Mol. Biol. Evol. 10: 1015–1029.

Nishi S. 1980. Differentiation of *Brassica* crops in Asia and the breeding of 'Hakuran', a newly synthesized leafy vegetable. In: Tsunoda S., Hinata K. and Gomez-Campo R.C. (eds), *Brassica* Crops and Wild Allies: Biology and Breeding. Jpn. Sci. Soc. Press, Tokyo, pp. 133–150.

Olsson G. 1954. Crosses within the *campestris* group of the genus *Brassica*. Hereditas 40: 398–418.

Oost E.H., Brandenburg W.A., Reuling G.T.M. and Jarvis C.E. 1987. Lectotypification of *Brassica rapa* L., *B. campestris* L. and neotypification of *B. chinensis* L. (Cruciferae). Taxon 36: 625–634.

Prakash S. and Hinata K. 1980. Taxonomy, cytogenetics and origin of crop *Brassica*, a review. Opera Bot. 55: 1–57.

Saitou N. and Nei M. 1987. The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Sinskaja E.N. 1928. The oleiferous plants and root crops of the family Cruciferae. Bull. Appl. Bot. Gen. Plant Breed. 19: 1–648.

Song K.M., Osborn T.C. and Williams P.H. 1988. *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs) 2. Preliminary analysis of subspecies within *B. rapa* (syn. *campestris*) and *B. oleracea*. Theor. Appl. Genet. 76: 593–600.

Song K., Osborn T.C. and Williams P.H. 1990. *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs) 3. Genome relationships in *Brassica* and related genera and the origin of *B. oleracea* and *B. rapa* (syn. *campestris*). Theor. Appl. Genet. 79: 497–506.

Tsunoda S. 1980. Eco-physiology of wild and cultivated forms in *Brassica* and allied genera. In: Tsunoda S., Hinata K. and Gomez-Campo R.C. (eds), *Brassica* Crops and Wild Allies: Biology and Breeding. Jpn. Sci. Soc. Press, Tokyo, pp. 109–120.

Van de Peer Y. and De Wachter R. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. 10: 569–570.

Vos P., Hongers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. and Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407–4414.