荞麦属植物淀粉酶和甲酸脱氢酶同功酶研究

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摘 要: 以聚丙烯酰胺凝胶电泳方法研究了荞麦属植物 8 个种 42 个收集系干种子和发芽种子的淀粉酶和甲酸脱氢酶同功酶。结果表明,荞麦淀粉酶在干种子中缺乏活性,但是在发芽种子中活性很强。在供试材料的发芽种子中共发现 23 个淀粉酶谱带,其中甜荞和苦荞分别有 10 条和 8 条。不同荞麦种间淀粉酶谱带差异很大,但是同种内不同收集系间差异较小。谱带聚类分析表明大野荞和毛野荞分别与甜荞和苦荞较近缘,支持它们分别为甜荞和苦荞祖先种的假说。在干种子和发芽种子中,发现所有荞麦种类均只有 1 条位置一致的甲酸脱氢酶谱带,暗示该酶在进化中具有高度稳定性。

关键词: 普通荞麦; 苦荞; 淀粉酶; 甲酸脱氢酶; 系统关系; 起源与进化

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Amylase and formate dehydrogenase isozymes in the genus *Fagopyrum*

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Abstract: The amylase isozyme and formate dehydrogenase isozyme of 42 accessions of cultivated and wild buckwheat belonging to eight species of the genus *Fagopyrum* including seven species of the big-achene group and one of the small-achene group were studied by means of polyacrylamide gel electrophoresis(PAGE). The isozyme samples were obtained from dry and sprouting seeds, respectively. The results showed that dry seeds had no amylase activity but the sprouting seeds had very active amylase isozymes. A total of 23 bands for the amylase isozyme were observed in sprouting seeds of cultivated and wild buckwheat, with *F. esculentum* and *F. tataricum* having 10 and 8 bands, respectively. Variations in the amylase isozyme zymographs were large among different buckwheat species but were less so within the same species. The results of zymograph analysis and clustering showed that *F. megaspartanium* and *F. pilus* are closely related to *F. esculentum* and *F. tataricum*, respectively, supporting Chen's hypothesis that *F. megaspartanium* and *F. pilus* are ancestors of these two species, respectively. Only one band was observed for the formate dehydrogenase isozyme in the dry and spouting seeds of all accessions.

Key words: common buckwheat; tartary buckwheat; amylase isozyme; formate dehydrogenase isozyme; phylogeny; origin and evolution

Isozyme formation occurs when a living organism adapts to its environment during the evolutionary process. Isozymes show specificity in different species and genera, different tissues and organs, and during different de-

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velopmental stages (Hu & Wan, 1985; Bao & Chen, 1993; Alekhova *et al.*, 2001; Qu *et al.*, 2003; Liu *et al.*, 2004; Yang *et al.*, 2006). Using isozyme zymographs, it is possible to determine gene existence, examine gene expression, and infer gene hereditary. At present, enzyme isozymes are widely applied as genetic and biochemical markers in studies of species identification, origin, evolution, and classification in many fields. A number of studies have shown that the amylase isozyme is a particularly useful tool in phylogenetic studies and species identification (Yuan & Liang, 1993; Wang *et al.*, 2000; Zhao *et al.*, 2000; Jiang & Pen, 2002; Hu *et al.*, 2003; Xie *et al.*, 2004). Formate dehydrogenase, on the other hand, although it widely exists in higher plants, has scarcely been reported.

Buckwheat, of which there are about 16 species (Chen, 1999a, b; Chen *et al.*, 2004), belongs to the genus *Fagopyrum* of the family Polygonaceae (Wu, 1983; Lee *et al.*, 1994; Chen, 2001a, b; Wang *et al.*, 2005, 2006). Buckwheat species are often classified into two groups, the big-achene and the small-achene group, respectively. The former includes two cultivated species (*F. esculentum* and *F. tataricum*), four natural wild species (*F. zuogongense* Q. F. Chen, *F. megaspartanium* Q. F. Chen, *F. pilus* Q. F. Chen and *F. cymosum* Meissn) and one man-made species (*F. giganteum* Krotov) (Ye & Guo, 1992; Ohnishi & Matsuoka, 1996; Chen, 1999a, b), while the latter includes nine wild species, *F. gracilipes* (Hemsl.) Dammer ex Diels, *F. leptopodum* (Diels) Hedberg, F. statice Gross, *F. capillatum* Ohnishi, *F. callianthum* Ohnishi, *F. gilesii* (Hemsl) Hedberg, *F. pleioramosum* Ohnishi, *F. lineare* (Sam.) Haraldsom and *F. urophyllum* Gross (Chen, 1999a, b). There are significant genetical and morphological differences between these two groups (Chen, 1999a, b; Chen, 2001; Chen et al, 2004).

It is generally believed that *F. cymosum* (= *F. cymosum* complex) is the ancestor of cultivated buckwheat, as supported by buckwheat morphology (Hedberg, 1946; Campbell, 1976). However, based on the apparent variations among different populations of *F. cymosum* (= *F. cymosum* complex) in, for example, morphology, cytology, reproduction isolation and isozymes, Chen (1999a, b) and Chen *et al.* (2004) classified this species into three separate biological species: the perennial diploid *F. megaspartanium*, perennial diploid *F. pilus*, and perennial allotetraploid *F. cymosum*, collectively referred to as the *F. cymosum* complex. In addition, they suggested that *F. megaspartanium* and *F. pilus* are the ancestors of common buckwheat and tartary buckwheat, respectively.

To date, no reports have examined the amylase and formate dehydrogenase isozymes in the genus *Fagopyrum*. In the present study, we examined the presence of these two isozymes in all buckwheat species of the big-achene group and some of the small-achene group at different stages of development, in order to provide clues and new data regarding the interspecific phylogeny of *Fagopyrum* as well as the origin and evolution of cultivated buckwheat, and to provide important information for buckwheat genetics and breeding.

1 Materials and Methods

Forty-two buckwheat accessions belonging to eight buckwheat species were used in this study (Tables 1 and 2). All accessions were provided by the Institute of Plant Genetics and Breeding where all studies were carried out.

1.1 Preparation of amylase (AMY) and formate dehydrogenase (FDH, E. C. 1. 2. 1. 2) isozyme samples

The preparation of isozyme samples from sprouting seeds largely followed the methods of Chen (1999a) and Hu (1985). Briefly, buckwheat seeds were placed on humid filter paper in a dish and cultured at 25 ℃ in an illumination incubator. When the roots were about 2. 0 cm long, 0. 5 g of sprouting seeds were ground in extracting solution [0. 9 g polyvinylpolypyrrolidone (PVPP), 0. 5 g silicon dioxide, and 0. 8 mL 0. 1 mol/L Tris-HCl buffer, pH 7. 5] into uniform juice in a mortar on ice then centrifuged at 9 167 *g* at 4 ℃ for 20 min. The supernatant solution was used as the isozyme sample for electrophoresis.

The preparation of isozyme samples from dry seeds largely followed the method of Chen (2004). The main procedures were as follows: 0. 3 g dry buckwheat seed powderwere placed in a mortar with 0. 9 mL 0. 1 mol/L Tris-HCl buffer (pH7. 5), 0. 5 g silicon dioxide, and 0. 96

Table 1　Cultivated buckwheat types and man-made buckwheat accessions used in this study

Accession	Species	Name	Ploidy	Native to	Symbol
ES2004010101	*F. esculentum*	Bijie Tianqiao	2x	Bijie, Guizhou	ES1
ES2004102902	*F. esculentum*	Qianxi Tianqiao	2x	Qianxi, Guizhou	ES2
ES2004010201	*F. esculentum*	Weining Tianqiao	2x	Weining, Guizhou	ES3
ES2004091702	*F. esculentum*	Shuicheng Tianqiao	2x	Shuicheng, Guizhou	ES4
ES2004091703	*F. esculentum*	Zunyi Tianqiao	2x	Zunyi, Guizhou	ES5
ES2004091701	*F. esculentum*	Daozhen Tianqiao	2x	Daozhen, Guizhou	ES6
ES2003110102	*F. esculentum*	Suiyang Tianqiao	2x	Suiyang, Guizhou	ES7
ES2004010401	*F. esculentum*	Xingren Tianqiao	2x	Xingren, Guizhou	ES8
ES2004030101	*F. esculentum*	Pingqiao 2	2x	Liangshan, Sichuan	ES9
ES2004102901	*F. esculentum*	Hunan Tianqiao	2x	Wugang, Hunan	ES10
ES2004062001	*F. esculentum*	Sibano	2x	Germany	ES11
ES2004082201	*F. esculentum*	Jieke Tianqiao	2x	Czech Republic	ES12
TA2003120101	*F. tataricum*	Bijie Kuqiao	2x	Bijie, Guizhou	TA1
TA2003120102	*F. tataricum*	Weining Kuqiao. 2	2x	Weining, Guizhou	TA2
TA2003120103	*F. tataricum*	Weining Kuqiao 3	2x	Weining, Guizhou	TA3
TA2001112202	*F. tataricum*	Kuchiqiao	2x	Weining, Guizhou	TA4
TA2004041507	*F. tataricum*	Qianhei 1	2x	Weining, Guizhou	TA5
TA2004041503	*F. tataricum*	Guizhou Kuqiao 2	2x	Weining, Guizhou	TA6
TA2004081103	*F. tataricum*	Guizhou Kuqiao 4	2x	Weining, Guizhou	TA7
TA2001112203	*F. tataricum*	Laoya Kuqiao	2x	Weining, Guizhou	TA8
TA2004100102	*F. tataricum*	Shuicheng Kuqiao	2x	Shuicheng, Guizhou	TA9
TA2001100501	*F. tataricum*	Yanhe Kuqiao 1	2x	Yanhe, Guizhou	TA10
TA1998100101	*F. tataricum*	Yanhe Kuqiao 2	4x	Man-made	TA11
TA2004081101	*F. tataricum*	Jiujiang Kuqiao	2x	Jiujiang, Sichuan	TA12
TA2004102902	*F. tataricum*	Hunan Kuqiao	2x	Wugang, Hunan	TA14
TA2003100801	*F. tataricum*	Shanxi Kuqiao	2x	Datong, Shanxi	TA15
GI2003032001	F. giganteum	Gig-1	4x	Man-made	GI1
GI2003080101	F. giganteum	Gig-2	4x	Man-made	GI2

Table 2　Wild buckwheat accessions used in the study

Accession	Species	Ploidy	Native to	Symbol
HO2004101901	*F. esculentum* var. *homotropicum*	2x	Yunnan	HO1
CY2002062501	*F. cymosum*	4x	Yunnan	CY1
ZU2003070101	*F. zuogongense*	4x	Zuogong, Tibet	ZU1
PI2004120101	*F. pilus*	2x	Gongbujiangda, Tibet	PI1
PI2004120102	*F. pilus*	2x	Gongbujiangda, Tibet	PI2
ME2004100101	*F. megaspartanium*	2x	Dujiangyan, Sichuan	ME1
ME2004100201	*F. megaspartanium*	2x	Luzhou, Sichuan	ME2
ME2004100301	*F. megaspartanium*	2x	Loushanguan, Guizhou	ME3
ME2003101201	*F. megaspartanium*	2x	Guiyang, Guizhou	ME4
ME2003101202	*F. megaspartanium*	2x	Yuntaishan, Guizhou	ME5
ME2003101203	*F. megaspartanium*	2x	Leishan, Guizhou	ME6
ME2004090101	*F. megaspartanium*	2x	Lixian, Yunnan	ME7
ME2004120101	*F. megaspartanium*	2x	Xishan, Yunnan	ME8
GR2004100701	*F. gracilipes*	4x	Xishan, Yunnan	GR1

g PVPP, ground into uniform juice on ice then centrifuged at 10 000 rpm at 4 ℃ for 20 min. The supernatant solution was used as the isozyme sample for electrophoresis.

1.2 Electrophoresis and dyeing

Polyacrylamide gel electrophoresis (PAGE) with vertical plates was used for analysis of the amylase and formate dehydrogenase isozymes (Chen 1999a; Chen *et al.*, 2004). The main parameters were T = 8.5% (pH8.9) and C=0.3. The electrophoresis buffer used in this study was Tris-Gly(pH8.3). The amylase and formate dehydrogenase isozymes were dyed according to Hu

& Wan(1985).

Dyeing of amylase isozyme: the gel was placed in 100 mL acetic acid buffer solution(0. 15 mol/L, pH = 5. 0)1. 5 h at 37 ℃ then dyed in 100 mL dyeing solution (0. 005 g iodine and 0. 079 g potassium iodide) until white bands were showed visible. The gel was then washed with double distilled water and pictures were taken using a data camera.

Dyeing of formate dehydrogenase isozyme: the gel was placed in 100 mL dyeing solution (0. 42 g Tris (hydroxymethyl)aminomethane, 0. 14 g Sodium formate, 0. 03 g NAD Trihydrate, 0. 03 g NBT, and 0. 06 g PMS) for 1h under dark conditions at 25 ℃. The gel was then washed with double distilled water and pictures were taken using a data camera.

1. 3 Data analysis

Hierarchical cluster analysis and SPSS 11. 1 software were used for clustering the buckwheat accessions. Each sample was regarded as an accession. For a certain band, having the band noted one and otherwise zero. The Euclidean distance, $d(X_i, X_j)$, was used to estimate the distance between any two accessions(X_i and X_j):

$$d(X_i, X_j) = \sqrt{\sum_{k=1}^{m} (x_{ik} - x_{jk})^2}, i, j = 1, 2, 3, \cdots, n; k = 1, 2, 3, \cdots, m$$

where n = number of accessions and m = number of isozyme bands.

2 Results and Analysis

2. 1 Amylase isozyme

There are no bands of amylase isozymes in all dry seed samples but many bands in all sprouting seed samples, indicating that the amylase isozymes were not active in dry seeds for all accessions, but very active in sprouting seeds. Zymograms and idiograms of sprouting seeds from 25 buckwheat accessions belonging to eight buckwheat species are shown in Figs. 1 — 4. According to these figures, a total of 23 amylase isozyme bands were discovered in this study. The variations in the amylase isozyme zymograms were significant among different buckwheat species but were less so within the same species.

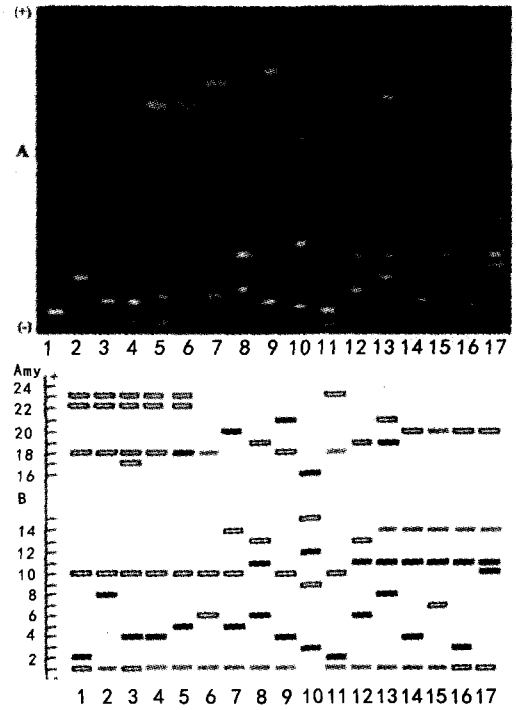

Fig. 1 Amylase isozyme zymographs(**A**)and ideograms (**B**)of sprouting seeds of buckwheat

1-17 is ES5, ES11, ES8, ES2, ES12, ZU1, PI1, GI1, ME4, GR1, HO1, GI2, CY1, TA7, TA11, TA5, and TA9, respectively. Dark rectangle = very dark band, grey rectangle = dark band, empty rectangle = light band.

A total of 10 bands(AMY-1, 2, 4, 5, 8, 10, 17, 18, 22, 23) were observed in sprouting seeds of *F. esculentum* accessions, with consensus bands AMY-1, 10, 18, 22, 23 and varible bands Amy-2, 4, 5, 8, 17 among accessions. *F. esculentum* var. *homotropicum*(HO1) and *F. zuogongense*(ZU1) had five bands(AMY-1, 2, 10, 18, 23) and four bands (AMY-1, 6, 10, 18), respectively, similar to common buckwheat. *F. megaspartanium* also had five bands(AMY-1, 4, 10, 18, 21), also similar to common buckwheat. A total of eight bands(AMY-1, 3, 4, 7, 10, 11, 14, 20) were observed in the *F. tataricum* accessions, of which there were four consensus bands(AMY-1, 11, 14, 20) and four varible bands(Amy-3, 4, 7, 10) among accessions. *F. pilus*(PI1) had AMY-1, 5, 10, 14, 20 bands, similar to *F. tataricum*. *F. giganteum*(GI1, GI2) had a total of five bands(AMY-1, 6, 11, 13, 19), different from *F. cymosum*(CY1) which had six bands(AMY-1, 8, 11, 14, 19, 21). According to these results, all big-achene group

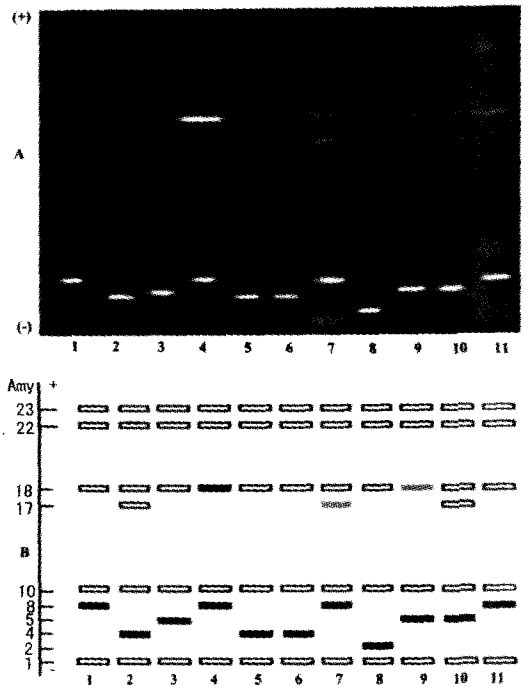

Fig. 2　Amylase isozyme zymographs(**A**)and ideograms (**B**)of sprouting seeds of *F. esculentum*

1-11 is respective ES1, ES8, ES12, ES7, ES4, ES2, ES10, ES5, ES6, ES3 and ES9. dark rectangle=very dark band, grey rectangle=dark band, empty rectangle=light band

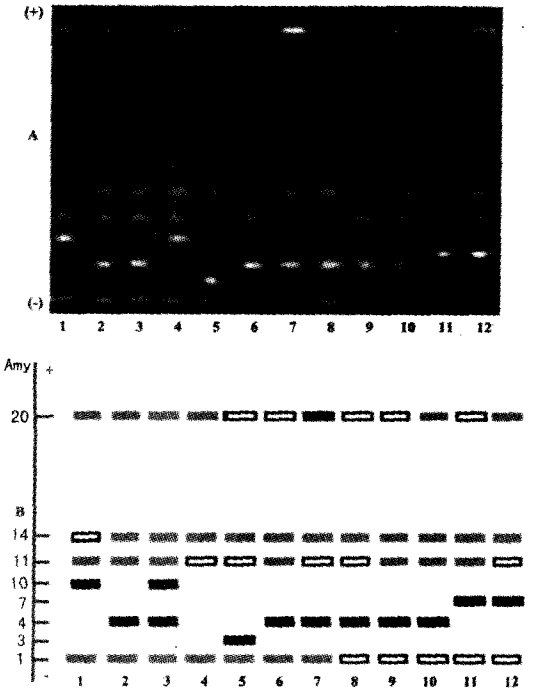

Fig. 3 Amylase isozyme zymographs(**A**)and ideograms (**B**) of sprouting seeds of *F. tataricum*

1-12 is respective TA9, TA4, TA10, TA15, TA5, TA8, TA6, TA7, TA12, TA2, TA14 and TA1. dark rectangle=very dark band, grey rectangle=dark band, empty rectangle=light band

buckwheat species had one consensus band(AMY-1). *F. gracilipes*(GR1), on the other hand, had a total of five bands(AMY-3,9,12,15,16), very different from the big-achene group of buckwheat species.

According to the distribution of bands, the amylase isozyme zymograms could be divided into three sections: a slow region(AMY-1-11), middle region(AMY-12-18) and fast region(AMY-19-23). The variation bands of common buckwheat all distributed at slow and middle regions and those of tartary buckwheat only at slow region. Further, according to the amylase isozyme zymograms of the different buckwheat species, four types could be determined: Type I with AMY-10 in the slow region and AMY-18 in the middle region(*F. esculentum*, *F. megaspartanium*, *F. zuogongense*, and *F. esculentum* var. *homotropicum*); Type II with AMY-14 in the middle region and AMY-20 in the fast region(*F. tataricum* and *F. pilus*); Type III with AMY-11 in the slow region and AMY-19 in the fast region(*F. giganteum* and *F. cymosum*); and Type IV with AMY-12,15,16 in the middle region(*F. gracilipes*(GR1)only).

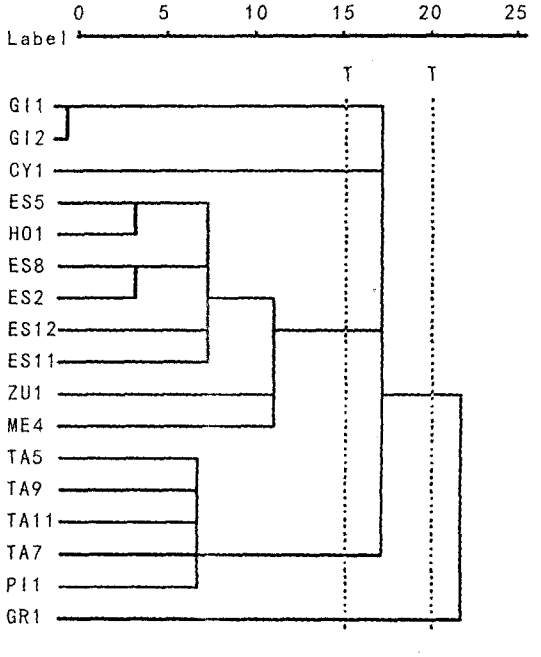

Fig. 4　Clustering tree of the buckwheat accessions based on amylase isozyme zymograms of sprouting seeds

The above buckwheat accessions were systematically clustered by means of Euclidean distance analysis(Fig. 4). The results showed that when T was equal to 15, the buckwheat species accessions could be divided into five groups:Group I and Group II included the tetraploid *F. giganteum* and *F. cymosum*, respectively; Group III, *F. esculentum*, *F. megaspartanium*, *F. zuogongense* and *F. esculentum* var. *homotropicum*; Group IV, *F. tataricum* and *F. pilus*; and Group V, *F. gracilipes*. When T was equal to 20, on the other hand, the buckwheat species accessions were grouped into two groups, that is, the big-achene group species and the small-achene group species, respectively, indicating that there is a distant relationship between these two groups.

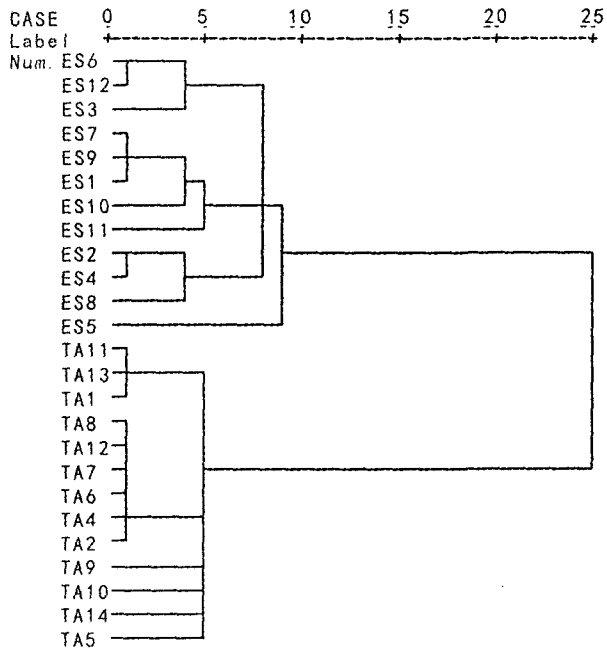

Fig. 5 Clustering tree of common buckwheat and tartary buckwheat accessions based on amylase isozyme zymograms of sprouting seeds

According to the Fig. 1, the isozyme band number of common buckwheat and tartary buckwheat in Fig. 2 and 3 are easy to be defined. The 25 accessions of common buckwheat and tartary buckwheat were systematically clustered by means of Euclidean distance and the nearest neighbor analysis of hierarchical classification (Fig. 5). It is clear that classifications for both of common buckwheat and tartary buckwheat have not obvious relationship with their geographic distribution. The accessions native to Czech Republic(ES12) and Germany (ES11) are close to those native to China, respectively.

2.2 Formate dehydrogenase isozyme

The formate dehydrogenase isozyme zymograms and idiograms of dry seeds and sprouting seeds from 25 accessions belonging to eight buckwheat species are shown in Figs. 6 and 7. According to these figures, all accessions had only one band, and no variations within species or among species were observed.

3 Discussion

3.1 Amylase isozyme in buckwheat and identification of buckwheat species

No previous report has documented the buckwheat amylase isozyme until now. The amylase isozyme of all buckwheat species of the big-achene group and some buckwheat species of the small-achene group was therefore studied for the first time in the present paper. The results showed that dry seeds have no amylase activity while sprouting seeds were very active. The sprouting seeds of *F. esculentum* accessions had 10 bands including 5 consensus bands and 5 variable bands while *F. tataricum* accessions had 8 bands including 4 consensus bands and 4 variable bands, less than common buckwheat. The variations in the amylase isozyme zymograms were also very large among different buckwheat species but were less so within the same species. All cultivated and wild buckwheat species used in this study had their own particular amylase isozyme zymogram, which can thus be used for identification of buckwheat species. There are about 16 species of buckwheat(Chen, 1999a, b), which can be grouped two sections, that is, the big-acheen group consisting of common buckwheat, tartary buckwheat, *F. zuogonense*, *F. megaspartanium*, *F. pilus*, *F. cymosum*, and *F. giganteum*; the small-achene-group consisting of 9 species(*F. gracilipes*, *F. leptopodum*, *F. urophyllum*, etc.). The *F. cymosum* complex consists of three similar species that cannot be easily distinguished. It may result in puzzled conclusions that the *F. cymosum* complex are confused as a species. Chen (1999a) and Chen et al(2004) reported two effective methods of buckwheat species identification by estaerase isozyme and

glutamate oxaloacetate transaminase isozyme The results in this study provide one more good tool for their identification. All species of the big-achene group had a consensus amylase band(AMY-1)different from that of the small achene group, which again can be used for distinguishing these two groups.

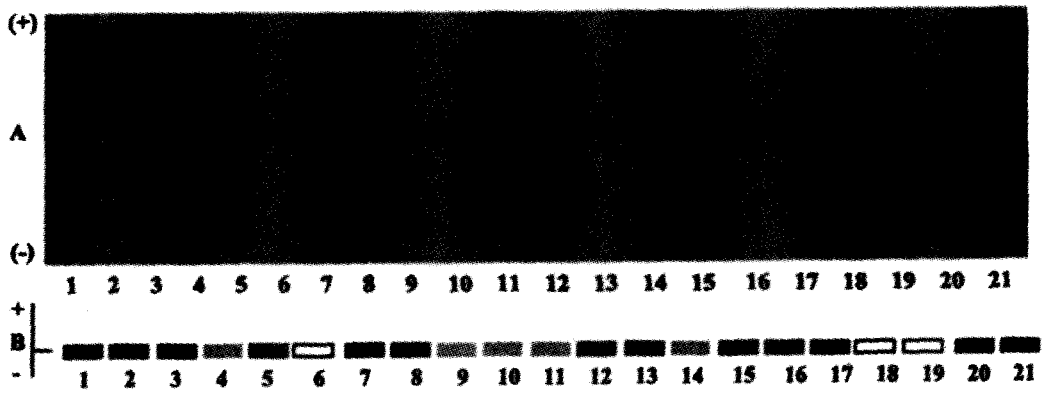

Fig. 6　Formate dehydrogenase isozyme zymographs(**A**)and ideograms(**B**)of dry seeds of buckwheat
1-21 are ES7, ES10, ES11, ES12, ZU1, PI1, GI1, GI2, ME1, ME2, ME3, ME4, ME5, ME6, ME7, GR1, HO1, CY1, TA3, TA11 and TA15, respective. Dark rectangle=very dark band, grey rectangle=dark band, empty rectangle=light band

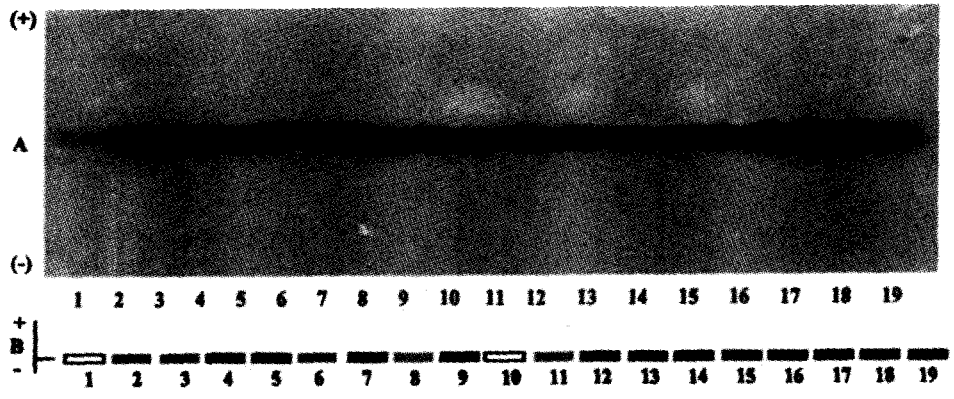

Fig. 7　Formate dehydrogenase isozyme zymographs(**A**)and ideograms(**B**)of sprouting seeds of buckwheat
1-19 is ES7, ES11, ES12, ZU1, PI1, GI1, GI2, ME1, ME2, ME3, ME4, ME5, ME6, GR1, HO1, CY1, TA3, TA11 and TA13, respective. Dark rectangle=very dark band, grey rectangle=dark band, empty rectangle=light band

3.2 Formate dehydrogenase isozyme in buckwheat

As above, no previous report has examined the formate dehydrogenase isozyme of buckwheat, and this study is the first to report this isozyme in dry and sprouting seeds of all species of the big-achene group. The results showed that activity of the formate dehydrogenase isozyme is high in both sprouting and dry seeds. Further, all accessions of *F. esculentum*, *F. tataricum*, and wild buckwheat had only one band at the same position, indicating that the formate dehydrogenase isozyme is highly conserved in the genus *Fagopyrum*.

3.3 Origin of cultivated buckwheat

Until recently, despite large variations, all perennial natural buckwheat types with a big achene were classified as *F. cymosum* and considered the proposed ancestor of cultivated buckwheat(*F. esculetum* and *F. tataricum*). Recently, however, Chen(1999a, b, 2001a, b)and Chen *et al.* (2004) put forward the concept of the *F. cymosum* complex, classifying this single species into three different biological species, the diploids *F. megaspartanium* and *F. pilus* and allotetraploid *F. cymosum*. In addition, the morphology, chromosome karyotype, esterase isozyme, glutamate oxaloacetate transaminase (GOT) isozyme, interspecific crossability and reproduction properties(Chen, 1999a, b, 2001a, b; Chen *et al.*, 2004)all indicated that *F. megaspartanium* and *F. pilus* are the an-

cestor species of *F. esculentum* and *F. tataricum*, respectively.

Yamane & Ohnishi(2001) reported that the phylogenetic tree constructed using the neighbour-joining method based on allozyme variation clarified two distinct groups of diploid populations of the *F. cymosum* complex. Further, Yamane *et al.* (2003) showed that *F. tataricum* is similar to one member(maybe *F. pilus*) of the *F. cymosum* complex based on cpDNA variability, suggesting that *F. tataricum* diverged from this species in the Tibet-Himalayan area. Collectively, these reports strongly support Chen's(1999a) classification of *F. pilus* and *F. megarspartanium* as separate species and the hypotheses that they are ancestors of cultivated buckwheat. In this study, *F. esculentum* and *F. tataricum* had very similar amylase isozyme zymographs to *F. megaspartanium* and *F. pilus*, respectively, further supporting the above origin hypothesis suggested by Chen(1999a, b).

The results in this study showed that accession classifications for both of common buckwheat and tartary buckwheat have not obvious relationship with their geographic distribution and that the accessions native to Czech Republic and Germany are close to those native to China, respectively, which agree to the hypothesis that common buckwheat and tartary buckwheat originated in southwestern China and then spread to oversea(Chen, 1999a, b; Ohnishi, 2007).

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