Doctoral Dissertation (Shinshu University)

Genetic Diversity and Phylogenetic Relationships in Grain Amaranths

子実用アマランサスの遺伝的多様性と系統分類に関する研究

March 2016

2016 年 3 月

Kazuhiro NEMOTO

根本 和洋

Interdisciplinary Graduate School of Science and Technology,
Shinshu University
Dept. of Biosci. & Food Product. Sci.

信州大学総合工学系研究科
生物・食料科学専攻

Genetic Diversity and Phylogenetic Relationships in Grain Amaranths

Kazuhiro NEMOTO

Synopsis

The genus *Amaranthus* consist of about 75 cultivated and wild species distributed throughout the world. Grain amaranths are noted for the high protein quality and quantity in their seeds and accordingly extensive research has been concluded in agronomical aspect of the development of this crop. However, only a few previous studies were concerned with the genetic diversity and phylogenetic relationships of *Amaranthus.* In this study, the *GBSSI* genes and their mutants from three species of grain amaranths were isolated and characterized. Comparison of the three *GBSSI* genes revealed a very high level of sequence conservation and structures that were similar to those of *GBSSI* genes in other plants. However, some regions of the amaranth *GBSSI* sequence (e.g. the transit peptide, including the cleavage site) had DNA and amino acid sequences that differed from those of other dicots. The waxy phenotype of three amaranth grains resulted from one base insertion in *GBSSI* in *Amaranthus caudatus* and a base substitution in *GBSSI* in *A. cruentus* and *A. hypochondriacus*. Each of these mutations added an internal termination codon into the *GBSSI* gene. Thus, a nonsense mutation (*A. cruentus* and *A. hypochondriacus*) or a frameshift mutation (*A. caudatus*) was responsible for the waxy phenotype. In addition, genetic resources collected from diverse regions were used to examine allelic diversity in amaranth *GBSSI* genes that affect amylose content. Finally, quantitative real-time PCR was used to examine the expression pattern of the *GBSSI* gene in seeds at different developmental and in several tissues. The results indicated that the amaranth *GBSSI* gene expressed late in seed development plays a role in the perisperm, and that it is expressed in both storage and non-storage organs.

Contents

Chapter 1

General Introduction

1.1 Structure and synthesis of starch

Starch is one of the major storage components in plants and is also an important factor in grain yield and quality. In plants, it serves as a primary store of carbon skeletons for metabolism and biosynthesis. It is synthesized both in chloroplasts of green plant tissues as transitory starch and in the amyloplasts of plant storage tissue as storage starch. Most of the starch in plants is composed of two distinct polymers: essentially linear amylose and highly branched amylopectin at a ratio of approximately 1:3. The starch biosynthesis process is summarized schematically in Fig. 1.1. Amylose is a lightly branched linear molecule with low polymerization, whereas amylopectin is a much larger molecule with extensive branches resulting from α-1,6 linkages (Smith et al. 1997). Amylose is synthesized by a granule-bound starch synthase (GBSS) encoded by the *GBSSI* (=*Waxy*) gene, whereas amylopectin biosynthesis is catalyzed by "soluble" starch synthases (SSS), starch branching enzymes (BE), and starch debranching enzymes (DBE). Both types of polymers are elongated by starch synthase, which catalyzes the transfer of glucose (Glc) from adenosine diphosphate (ADP)-Glc to the growing glucan chains via an α-1,4-linkage (Martin and Smith 1995). The properties of starch are determined by the ratio of amylose to amylopectin, and by the frequency of chain branching and the chain length of amylopectin (Manners, 1989).

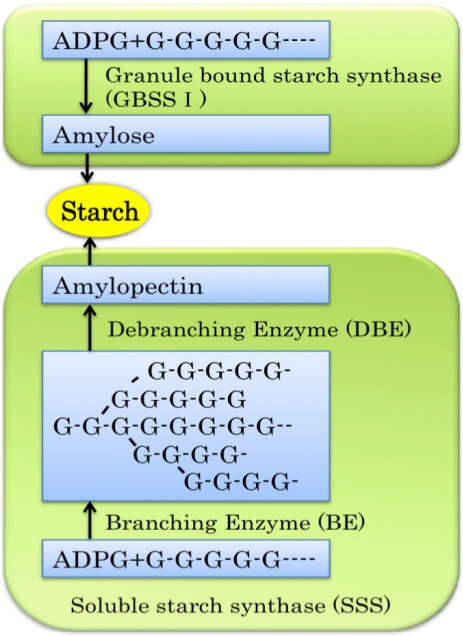


Fig. 1.1 Schematic representation of the starch biosynthesis pathway. The role of the various enzymes

Recently, several different starch synthases have been isolated and characterized from higher plants (Smith et al. 1997). There are at least six starch synthase isoforms,

namely GBSSI, GBSSII, SSSI, SSSII, SSSIII, and SSSIV, and several types of these enzymes have multiple isoforms, the number of which differs among plant species (Smith et al. 1997; Hirose and Terao 2004). Generally, GBSSI appears to be most common in storage organs, while GBSSII is responsible for amylose synthesis in leaves (Nakamura et al. 1998; Vrinten and Nakamura 2000). The roles of SSSI in starch biosynthesis are still poorly understood, although it is thought to play a role in the formation of the shortest glucan chains (Smith et al. 1997; Commuri and Keeling 2001). SSII and SSIII appear to be responsible for the elongation of amylopectin long chains (Smith et al. 1997). According to EST databases, SSIV is present in a wide range of higher plants, but to date, no specific role for this isoform has been identified (Tetlow et al. 2004b).

1.2 Granule bound starch synthase I

Granule-bound starch synthase I (GBSSI) is an important determinant of the amylose content of cereal starch, and is commonly referred to as the 'waxy protein' (Tsai 1974; Echt and Schwartz 1981; Okuno and Sakaguchi 1982; Sano et al. 1984; Konishi et al. 1985; Hovenkamp-Hermelink et al. 1987; Hseih 1988). The GBSSI protein is tightly bound to starch granules, and it has a molecular mass of 58–60 kDa (Salehuzzaman et al. 1993). The GBSSI protein is encoded at the *Waxy* locus in plants. This was one of the first plant genes cloned, and there is a large body of research on *GBSSI* expression, protein levels, activity, and its contribution to amylose synthesis in numerous species. Sequence information for *GBSSI* structural genes from several plants has been published. The *GBSSI* gene consists of 14 exons (Klosgen et al. 1986; Rohde et al. 1988; Wang et al. 1990; van the Leij et al. 1991; Okagaki 1992; Murai et al. 1999; Kimura et al. 2000; Fukunaga et al. 2002), and the amino acid sequence of the GBSSI protein shows strong similarities to those of other starch synthase isoforms (Ball et al. 1998, Kossman et al. 1999, Li et al. 1999).

While normal plant starches (non-waxy phenotype) are composed of 20–30% amylose and 70–80% amylopectin, waxy starches (waxy phenotype) contain little or no amylose (Fukunaga et al. 2002). Much of our understanding of waxy starches comes

from studies of mutants in many plant species lacking amylose. Several GBSSI mutants with unusual amylose/amylopectin ratios have been characterized (Davis et al. 2003). Maize *GBSSI* mutants were first shown to lack amylose in kernels (Sprague et al. 1943) and GBSSI in the endosperm (Nelson and Rines, 1962). Other *GBSSI* mutants have been identified in rice, barley, foxtail millet, common millet, sorghum, Job's Tears, and amaranth (Okuno and Sakaguchi 1982; Konishi et al. 1985; Wessler and Varagona 1985; Hseih 1988; Wang et al. 1995; Cai et al. 1998; Nakayama et al. 1998; Vrinten et al 1999; Domon et al. 2002a; Fukugawa et al. 2002). The mechanisms underlying these mutations have been characterized in some cereal species. The naturally occurring *GBSSI* mutations essentially eliminate or reduce amylose content of starch through the disrupted expression or decreased functioning of the *GBSSI* gene. In maize and foxtail millet, many spontaneous *GBSSI* mutations have been caused by transposable elements (Fedoroff et al. 1983; Wessler and Varagona 1985; Fukunaga et al. 2002). In rice, nucleotide substitutions have resulted in an aberrant 5′-end after splicing of intron 1 (Wang et al. 1995; Cai et al. 1998; Hirano et al. 1998). In wheat and barley, there are major deletions in the *GBSSI* loci (Vrinten et al. 1999; Domon et al. 2002a).

1.3 Grain amaranth and amaranth starch

The genus *Amaranthus* includes approximately 60 species that grow in many areas of the world. All *Amaranthus* are drought-resistant C4 photosynthetic plants that can grow well in saline, alkaline, acidic, or poor soil (Saunders and Becker 1984). This genus, which originated in the New World, is an ancient crop that was already under cultivation 5000–7000 years ago (Sauer 1967). Ancient amaranth grains still used to this day include the three species *Amaranthus caudatus*, *A. cruentus*, and *A. hypochondriacus* (Fig. 1.2). Today, amaranth is cultivated in Central and South America, Africa, India, and China (Saunders and Becker 1984). It was first introduced into Japan during the 1980s from the Rodale Research Institute in the United States. A new grain amaranth variety, “New Aztec”, was developed in 2001 using the “Mexico line”, which is a high-yielding semi-dwarf line selected in Japan from the introduced genetic resources of grain amaranth (*A. cruentus*) originating from Mexico (Katsuta et

al. 2001). Currently, cultivation of this new grain, mostly *A. cruentus*, is gradually increasing in some areas in Japan, including Iwate, Akita, and Nagano Prefectures.

The potential of both grain (seed) and vegetable (leaf) amaranths as food resources has been reviewed extensively (e.g. Becker et al. 1981; Morales et al. 1988; Shukla et al. 2006). Amaranth grains are widely used in a variety of food products including breakfast cereals, soup, breads, pancakes, cookies, and as an ingredient in confections. It also can be popped like popcorn, expanding to about 10 times its original volume. The popped amaranth has a toasted, nutty flavor, and can be used in a variety of ways. Amaranth leaves are an excellent source of carotene, iron, calcium, protein, vitamin C, and other trace elements. They can be used similarly to spinach, and can be boiled or fried as a tasty side dish.

The interest in cultivation of amaranth increased when it was revealed that the seeds contain proteins with a high lysine content (Downton 1973). The other biological interest is the unique characteristics of its starch. Like endosperm starch, amylose and amylopectin account for 48–62% of the weight of amaranth starch. The starch is located in the cells of the perisperm, where it forms very small starch granules (1–2 μm in diameter) compared with those of rice starch (3–8 μm in diameter) (Marcone 2001). The perisperm is in the center of amaranth seed, and is surrounded by the embryo. The rest of the seed is endosperm (Okuno and Sakaguchi 1981). As compared to other plant starches, the physical properties of amaranth starch suggest a high proportion of short-chain molecules in the amylopectin. In addition, compared with wheat and maize starches, the starch of amaranths has a much lower amylose content, lower swelling power, higher solubility, greater water uptake, lower amylograph viscosity, and greater gelatinization temperature range (Lorenz 1981; Stone and Lorenz 1984). It is likely to prove useful for applications in the food, plastics, cosmetics, and other industries.

The starch found in *Amaranthus* endosperm can be either nonwaxy or waxy. Okuno and Sakaguchi (1982) reported that starch grains from the perisperm of nonwaxy seeds are non-glutinous and contain amylase and amylopectin, whereas those from waxy seeds are glutinous and contain almost entirely amylopectin. The amylose content is 7–14% (nonwaxy) and 0% (waxy) in the perisperm of seeds. Amylose synthesis is

controlled by a single major structural gene, with the *waxy* allele recessive to the *Waxy* allele (Okuno and Sakaguchi 1981; Sugimoto et al. 1981; Konishi et al. 1985). These findings substantiated for the first time the presence of both nonwaxy and waxy phenotypes in a dicot species. However, the molecular basis of the *GBSSI* (=*Waxy*) gene and/or mutants of this trait in grain amaranths remained unknown.

Like in other cereals, the amylose content of the perisperm of grain amaranth plays an important role in palatability and starch quality. Thus, it is important to understand the genetic control of starch composition in amaranth seeds to design breeding and/or genetic engineering strategies aimed at enhancing quality. In this respect, a detailed understanding of the *GBSSI* gene and/or its mutants is required.

1.4 Aim and outline of this thesis

The aim of this research was to facilitate further studies on amaranth starch and/or those aimed at developing amaranth lines with diverse starch composition. For such studies, a thorough understanding of the genetic characteristics of *GBSSI* and the molecular mechanisms of its mutants is required. The amylose content of the seed and plant tissues in amaranth plays an important role in palatability and starch quality. The potential of amaranth resides in its naturally amylose-free or low amylose grain, which is a result of mutations that disrupt expression or result in loss of function of the *GBSSI* gene. Since the conformational structure of a molecule is responsible for its functional properties, it is essential to try to understand the structure-function relationship of amaranth starch. Thus, this thesis is a summary of scientific research on the genetic and molecular basis of amaranth starch.

To facilitate further studies on amaranth starch, it is essential to characterize the *GBSSI* gene and to establish its primary structures. The inheritance of *waxy* in grain amaranth (*A. cruentus*) perisperm was studied using reciprocal crosses. The complete coding sequence of the amaranth *GBSSI* was determined and its genomic structure was analyzed. These results are presented in Chapter 2.

To clarify the molecular basis of the *waxy* mutants' trait in grain amaranths, the structural variations between *Waxy* (non-waxy) and *waxy* (waxy) alleles in the coding

region of each of the three species were investigated. These results are discussed in Chapter 3.

Polymorphisms at the *Waxy* locus can provide useful information about the origins and evolutionary history of this gene. The evolution of the waxy phenotype in grain amaranth is presented in Chapter 4. These analyses were based on molecular phylogenetic analyses based on polymorphisms in a large gene pool of the *Waxy* locus.

A key aspect of understanding the mechanism of amylose synthesis in amaranth grain is to understand the relationship between the expression patterns of the *GBSSI* gene and amylose accumulation. Expression patterns of *GBSSI* in seeds at different developmental stages and in various tissues of amaranth were examined, and the results are presented in Chapter 5.

A general discussion of these findings and the implications for future research are presented in Chapter 6.

1.5 References

Ball SG, van de Wal MHBJ, Visser RGF (1998) Progress in understanding the biosynthesis of amylose. Trends Plant Sci 3:462-467

Becker R, Wheeler E, Lorenz K, Stafford A, Grosjean O, Betschart A, Saunders R (1981) A compositional study of grain amaranth. J Food Sci 46:1175-1180

Cai X, Wang Z, Xing Y, Zhang J, Hong M (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5'UTR and decreased expression of *waxy* gene in rice cultivars of intermediate amylose content. Plant J 14:459-465

Commuri PD, Keeling PL (2001) Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. Plant J 25:475-486

Davis J, Supatcharee N, Khandellwal R, Chibbar R (2003) Synthesis of novel starches in Planta: Opportunities and challenges. Starch/Staerke 55:107-120

Domon E, Fujita M, Ishikawa N (2002a) The insertion/deletion polymorphisms in the *waxy* gene of barley genetic resources from East Asia. Theor Appl Genet 104:132-138

Downton W (1973) *Amaranthus edulis*: A high lysine grain amaranth. World Crops

25:20

Echt C, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. Genetics 99:275-284

Fedoroff N, Wessler S, Shure M (1983) Isolation of the transposable maize controlling elements Ac and Ds. Cell 35:235-242

Fukunaga K, Kawase M, Kato K (2002) Structural variation in the *Waxy* gene and differentiation in foxtail millet [*Setaria italica* (L.) P. Beauv.]: implications for multiple origins of the waxy phenotype. Mol Genet Genomics 268:214-222

Hirano HY, Eiguchi M, Sano Y (1998) A single base change altered the regulation of the *waxy* gene at the posttranscriptional level during the domestication of rice. Mol Biol Evol 15:978-987

Hirose T, Terao T (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). Planta 220:9-16

Hovenkamp-Hermelink J, Jacobsen E, Ponstein A, Visser R, Vos-Scheperkeuter G, Bijmolt E, Vries J, Witholt B, Feenstra W (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor Appl Genet 75:217-221

Hseih J (1988) Genetic studies of the *Wx* gene of sorghum (*Sorghum bicolor* [L.] Moench). Bot Bull Academia Sinica 29:293-299

Katsuta M, Komeichi M, Okuyama Y, Honda Y, Shirato S, Nakatani M (2001) A new grain amaranth variety "New Aztec". Bulletin of the National Institute of Crop Science 1:57-70 (in Japanese)

Konishi Y, Nojima H, Okuno K, Asaoka M, Fuwa H (1985) Characterization of starch granules from waxy, nonwaxy, and hybrid seeds of *Amaranthus hypochondriacus* L. Agri Biol Chem 4:1965-1971

Kossmann J, Abel GJW, Springer F, Lloyd JR, Willmitzer L (1999) Cloning and functional analysis of a cDNA encoding a starch synthase from potato (*Solanum tuberosum* L.) that is predominantly expressed in leaf tissue. Planta 208:503-511

Klosgen R, Gierl A, Schwartz-Sommer Z, Saedler H (1986) Molecular analysis of the waxy locus of maize. Mol Gen Genet 203:237-244

Kimura T, Ideta O, Saito A (2000) Identification of the gene encoding granule-bound starch synthase I in sweet potato (*Ipomoea batatas* (L.) Lam.). Plant Biotechnol 17:247-252

Li Z, Chu X, Mouille G, Yan L, Kosar-Hashemi B, Hey S, Napier J, Shewry P, Clarke B, Appels R, Morell MK, Rahman S (1999) The localization and expression of the class II starch synthases of wheat. Plant Physiol 120:1147-1156

Lorenz, K. 1981. *Amaranthus hypochondriacus*-characteristics of the starch and baking potential of the flour. Starch 33:149

Marcone M (2001) Starch properties of *Amaranthus pumilus* (seabeach amaranth): a threatened plant species with potential benefits for the breeding/amelioration of present amaranthus cultivars. Food Chem 73:61-66

Martin C, Smith A (1995) Starch biosynthesis. Plant cell 7:971-985

Manners DJ (1989) Recent developments in our understanding of amylopectin structure. Carbohydr Polym 11:87-112

Morales E, Lembcke J, Graham G (1988) Nutritional value for young children of grain amaranth and maize-amaranth mixtures: effect of processing. J Nutri 118:78

Murai J, Taira T, Ohta D (1999) Isolation and characterization of the three *Waxy* genes encoding the granule-bound starch synthase in hexaploid wheat. Gene 234:71-79

Nakamura T, Vrinten P, Hayakawa K, Ikeda J (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol 118:451-459

Nakayama H, Afzal M, Okuno K (1998) Intraspecific differentiation and geographical distribution of *Wx* alleles for low amylose content in endosperm of foxtail millet, *Setaria italica* (L.) Beauv. Euphytica 102:289-293

Nelson O, Rines H (1962) The enzymatic deficiency in the *waxy* mutant of maize. Biochem Biophys Res Commun 9:297-300

Okagaki R (1992) Nucleotide sequence of a long cDNA from the rice *waxy* gene. Plant Mol Biol 19:513-516

Okuno K, Sakaguchi S (1981) Glutinous and non-glutinous starches in perisperm of grain amaranthus. Cereal Res Commun 9:305-310

Okuno K, Sakaguchi S (1982) Inheritance of starch characteristics in perisperm of *Amaranthus hypochondriacus*. J Heredity 73:467

Rohde W, Becker D, Salamini F (1988) Structural analysis of the waxy locus from *Hordeum vulgare*. Nucl Acids Res 16:7185-7186

Salehuzzaman S, Jacobsen E, Visser R (1993) Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato. Plant Mol Biol 23:947-962

Sano Y (1984) Differential regulation of *waxy* gene expression in rice endosperm. Theor Appl Genet 68:467-473

Sauer JD (1950) The grain amaranths: a survey of their history and classification. Ann Missouri Bot Gard 37:561-619

Sauer JD (1967) The grain amaranths and their relatives: a revised taxonomic and geographic survey. Ann Missouri Bot Gard 54:103-137

Saunders R, Becker R (1984) Amaranthus: a potential food and feed resource. Adv Cereal Sci Technol 6:357-396

Shukla S, Bhargava A, Chatterjee A, Srivastava J, Singh N, Singh S (2006) Mineral profile and variability in vegetable amaranth (*Amaranthus tricolor*). Plant Foods Hum Nutr 61:23-28

Smith AM, Denyer K, Martin C (1997) The synthesis of the starch granule. Annu Rev Plant Biol 48:67-87

Sprague G, Brimhall B, Hixon R (1943) Some effects of the *waxy* gene in corn on properties of the endosperm starch. J Am Soc Agron 35:817

Stone LA, Lorenz K (1984) The starch of amaranthus-physicochemical properties and functional characteristics Starch 36:232

Sugimoto Y, Yamada K, Sakamoto S, Fuwa H (1981) Some properties of normal-and waxy-type starches of *Amaranthus hypochondriacus* L. Starch/Staerke 33:112-116

Tetlow IJ, Wait R, Lu ZX, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ (2004b) Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. Plant cell 16:694-708

Tsai CY (1974) The function of the waxy locus in starch synthesis in maize endosperm. Biochem Genet 11:83-96

van der Leij F, Visser R, Ponstein A, Jacobsen E, Feenstra W (1991) Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. Mol Gen Genet 228:240-248

Vrinten P, Nakamura T, Yamamori M (1999) Molecular characterization of waxy mutations in wheat. Mol Genet Genomics 261:463-471

Vrinten PL, Nakamura T (2000) Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol 122:255-263

Wang Z, Wu Z, Xing Y, Zheng F, Guo X, Zhang W, Hong M (1990) Nucleotide sequence of rice *waxy* gene. Nucl Acids Res 18:5898

Wang Z, Zheng F, Shen G, Gao J, Snustad D, Li M, Zhang J, Hong M (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. Plant J 7:613-622

Wessler S, Varagona M (1985) Molecular basis of mutations at the *waxy* locus of maize: correlation with the fine structure genetic map. Proc Natl Acad Sci 82:4177-4181

Chapter 2

Variation and Geographical Distribution in Perisperm Starch of Grain Amaranths (*Amaranthus* spp.), and the Origin of Waxy Perisperm Type

2-1. Introduction

The starch-rich seeds of grain amaranths (*Amaranthus* spp.), which belong to the family Amaranthaceae in the Dicotyledoneae, are used as cereals, as well as quinoa and buckwheat grain. The seed storage starch of grain amaranths is stored in the diploid perisperm (2n), which is a modified nucellus of the embryo sac, while in the grass family (Gramineae) starch stored in cereal grains is deposited in the triploid endsperm (3n). They have three cultivated species i.e. *A. hypochondriacus* L., *A. cruentus* L. and *A. caudatus* L., all of which were domesticated from early antiquity in the New World (Sauer 1976).

Starch is a complex polysaccharide composed of amylose and amylopectin molecules. Non-waxy (non-glutinous) starch contains both amylose and amylopectin molecules, while waxy (glutinous) is composed of only amylopectin, or amylopectin with a very little amylose. In grain amaranths, the perisperm starch has waxy type, which was differentiated from non-waxy type by spontaneous mutation (Sakamoto 1997). This property has only been found in the Dicotyledoneae, in other words, except for cereals such as rice, foxtail millet, common millet, Job's tears, sorghum, barley and maize.

In previous reports, waxy type of a grain amaranth, which was stained reddish brown by iodine solution, has been referred in *A. leucosperma* S. Wats. (= *A. hypochondoriacus*) (Wolf et al. 1950) and *A. cruentus* (MacMasters *et al*. 1955). Since then, intraspesific differentiation of the types, non-waxy and waxy in *A. hypochondoriacus* (Sakamoto 1982), low-amylose type in *A. caudatus* (Tomita *et al*. 1980) and non-waxy, waxy and low-amylose type in *A. cruentus* (Inouchi *et al*. 1999) have been reported. Inheritance of the waxy perisperm trait is controlled by a single major structural gene with the waxy allele, which is recessive to the *Waxy* allele in *A.*

hypochondoriacus (Okuno and Sakaguchi 1981).

After the discovery of the New World by Columbus, the cultivation of grain amaranths was disseminated throughout Asia and Africa far away their place of origin. Especially, the southern foot of the Himalayas on the Indian subcontinent is regarded as the secondary center of genetic diversity (Sauer 1976), which contrasts markedly with the decline of amaranth cultivation in the New World.

The waxy/non-waxy trait, that is amylose content in starch granules in grains, is an important characteristic for breeding of cereal crops, particularly in rice, because differences in amylose content affects the texture of the grains processed. However, research on this in grain amaranths is limited. In addition, variation in the amylose content of these grain amaranths and the geographical distribution have not been revealed yet. Here, we describe the variation in amylose content and their geographical distribution in three grain species using amaranth genetic resources collected from around the world. We also discuss the origin of the waxy perisperm type in grain amaranths.

2-2. Materials and Methods

2-2-1. Plant Materials

A total of 266 accessions of grain amaranths, 97 of *A. hypochondriacus*, 99 of *A. caudatus*, and 70 of *A. cruentus*, collected from Central and South America, Asia, and Africa, were used in this study (Table 1). Among them, 219 strains were provided from the USDA-ARS National Plant Germplasm Amaranth Collection and 47 accessions of *A. hypochondriacus* and *A. caudatus* from around Asia, were collected by the authors. Some accessions consisted of mixtures of seeds with different seed coat color and/or appearance of the perisperm. In that case, seeds were separated and analyzed as a different accession.

2-2-2. Measurement of amylose content

Amylose content of perisperm starch was measured colorimetrically using a TECHNICON SOLIDPREPTM II autoanalyzer (Instruments Co., NY). Given the large

Table 1. Geographical distribution of perisperm types identified by I2-KI staining in three grain amaranth species

Origin	No. of strain	Perisperm type		
		Non-waxy	Low-amylose	Waxy
A. cruentus				
Central and South America				
Mexico	33	1	2	30
Guatemala	15	14	-	1
United States	6	1	-	5
Peru	3	3	-	-
Asia				
India	4	4	-	-
Africa				
Nigeria	2	2	-	-
Gana	3	3	-	-
Zambia	3	3	-	-
Zaire	1	1	-	-
Total	70	32	2	36
A. caudatus				
South America				
Peru	37	9	3	25
Bolovia	29	1	6	22
Argentin	7	6	1	-
Ecuador	1	1	-	-
Asia				
Nepal	18	-	18	-
India	5	-	3	2
Pakistan	1	-	1	-
Bhutan	1	-	1	-
Total	99	17	33	49
A. hypochondriacus				
Central and South America				
Mexico	46	35	-	11
United States	2	-	-	2
Guatemala	1	1	-	-
Puerto Rico	1	1	-	-
Brasil	1	-	-	1
Chile	1	-	-	1
Asia				
Nepal	22	2	-	20
Pakistan	6	-	-	6
India	5	-	-	5
China	5	-	-	5
Bhutan	2	-	-	2
Afganistan	1	-	-	1
Africa				
Nigeria	2	1	-	1
Uganda	1	-	-	1
Zambia	1	-	-	1
Total	97	40	-	57
Grand total	266	89	35	142

number of samples that had to be measured, whole grain powder instead of purified starch granules from the grains was used for the analysis. Twenty milligrams of seed powder passed through a 50-mesh screen was gelatinized in a mixture of 0.5N NaOH for more than two hours at room temperature, and then applied to the autoanalyzer. The amylose content was calculated using a calibration curve made from using values obtained from starch granule from *Oryza sativa* L. and *A. cruentus* (Table 2). Measurements on all accessions were conducted in triplicate.

Table 2. Standards of starch granule used to construct the calibration curve of amylose content.

	Variety /strain	Amylose content (%)
Oryza sativa L.	Mochiminori	0
	No. 1915	9.6
	Koshihikari	16.5
	Kochihibiki	21.5
A. cruentus	A.C.R104	0
	A.C.K112	15.8

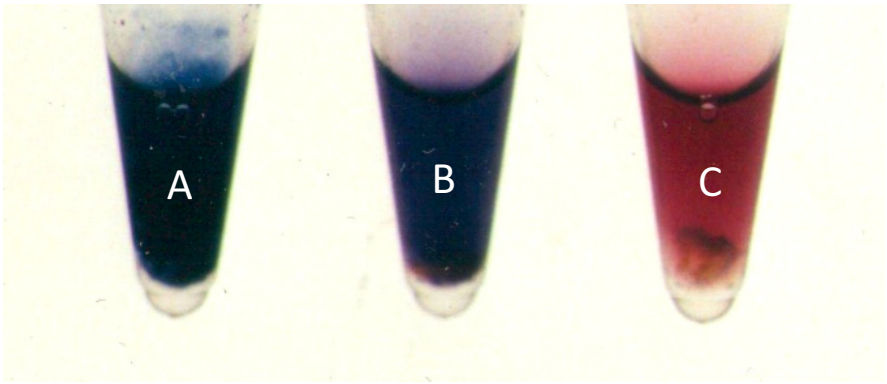


Fig. 1. Reaction of I_2-KI staining with different starch types. A: non-waxy type, B: low amylose type, C: waxy type.

2-2-3. Identification of perisperm starch type

Accessions were classified into three groups based on perisprm starch type, i.e. non-waxy, low-amylose and waxy, which could be clearly distinguished from one another by I2-KI staining; non-waxy, low-amylose and waxy types stained blue, purple and reddish brown, respectively (Fig. 1). In addition, these types could be also distinguished based on perisperm appearance, when the seed did not cover with seed coat colored by dark brown or black. Non-waxy and low-amylose types of perisperms were translucent, while non-waxy type of it was opaque, respectively (Fig. 2).

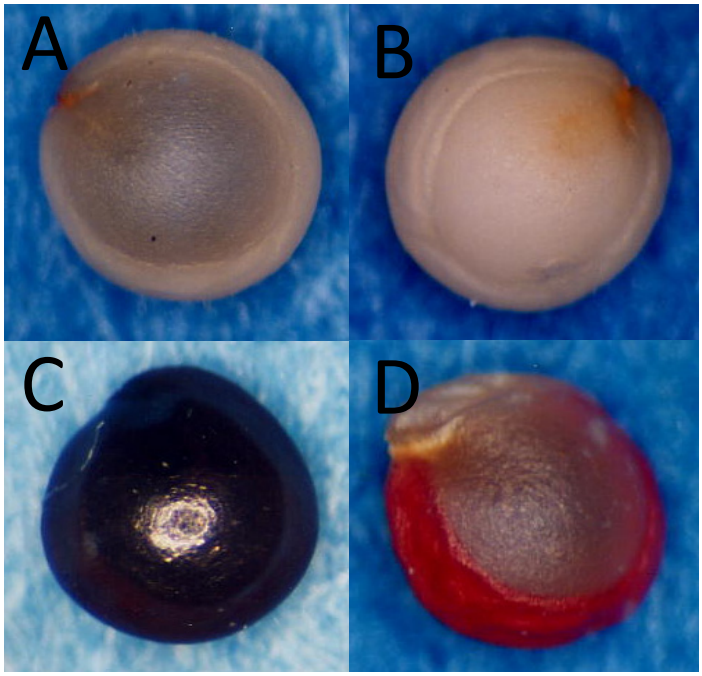


Fig. 2. Seed appearances of grain amaranths. A: non-waxy type of pale yellow seed. The perisperm is translucent (Center part). The low amylose type has same appearance like D. So that, both type can not be distinguished each other by the appearance. B: The waxy type of whitish yellow seed. The perisperm is opaque. It looks whiter than the non-waxy type (A). C: The seed is covered with dark brown seed coat. Perisperm type can not be identified into the non-waxy or waxy type by the appearance. D: The low amylose type of *A. caudatus*. Almost accessions in Asia have this appearance.

2-3. Results

2-3-1. Variation of amylose content and perisperm starch type

Amylose content of accessions used in this study varied from 0 to 17.7%. More specifically, *A. cruentus* showed a range from 0 to 12.0%, *A. hypochondoriacus* from 0 to 15.9%, and *A. caudatus* from 0 to 17.7%, respectively (Fig. 3). The range of amylose content in *A. cruentus* was thus slightly narrower than the range observed in the other two species. The frequency distribution of amylose content in *A. cruentus* and *A. hypochondoriacus* showed two clear peaks. In *A. caudatus*, frequency tended to decrease as amylose content increased.

I_2-KI staining and seeds appearance of *A. cruentus* and *A. caudatus* revealed the existence of three types of perisperm starch (waxy, low amylose and non-waxy) in these species (Table 1). On the other hand, the accessions of *A. hypochondoriacus* only contained two types (waxy and non-waxy). The three types, waxy, low-amylose and non-waxy ranged from 0 to 2.9%, 1.8 to 2.7% and 4.4 to 12.0% in *A. cruentus*, 0 to 1.9%, 0.9 to 5.9% and 8.0 to 17.7% in *A. caudatus*, respectively. In *A. hypochondoriacus*, the two types, waxy and non-waxy ranged from 0 to 2.7% and 6.8 to 15.9%, respectively. By the amylose content, which value measured by Autoanalyzer is relative, waxy and low amylose type could not be identified decidedly each other. Because the variation range of both types in two species overlapped, although both type can easily be identified by the appearance of perisperm (See Fig. 2). Among three types, the waxy type was the most dominated in all three species (Table 1). Whereas only 2 of 70 low-amylose accessions were found in *A. cruentus*, 33 of 99 strains were found in *A. caudatus*.

2-3-2. Geographical distribution in amylose content and perisperm starch type

The geographical distribution in amylose contents and perisperm types are shown in Fig. 4 and Table 1. Wide variation in both traits was observed in Central and South America where these species were domesticated. Opposite to this, limited variation was observed in Asia and Africa.

In *A. cruentus*, all of the accessions from Asia and Africa were of the

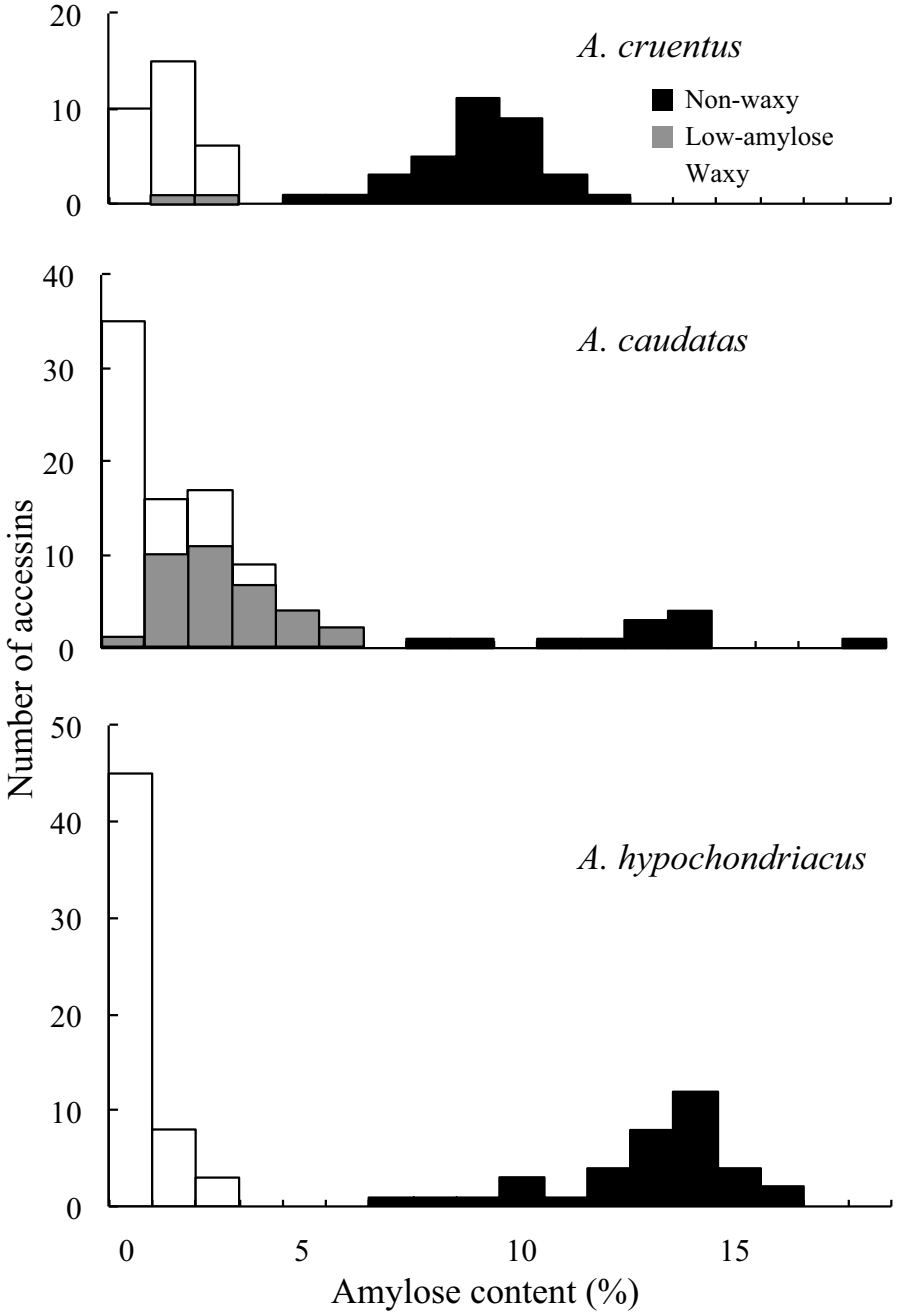


Fig.1. Frequency distribution of amylose content and waxy starch type in three grain amaranth species.

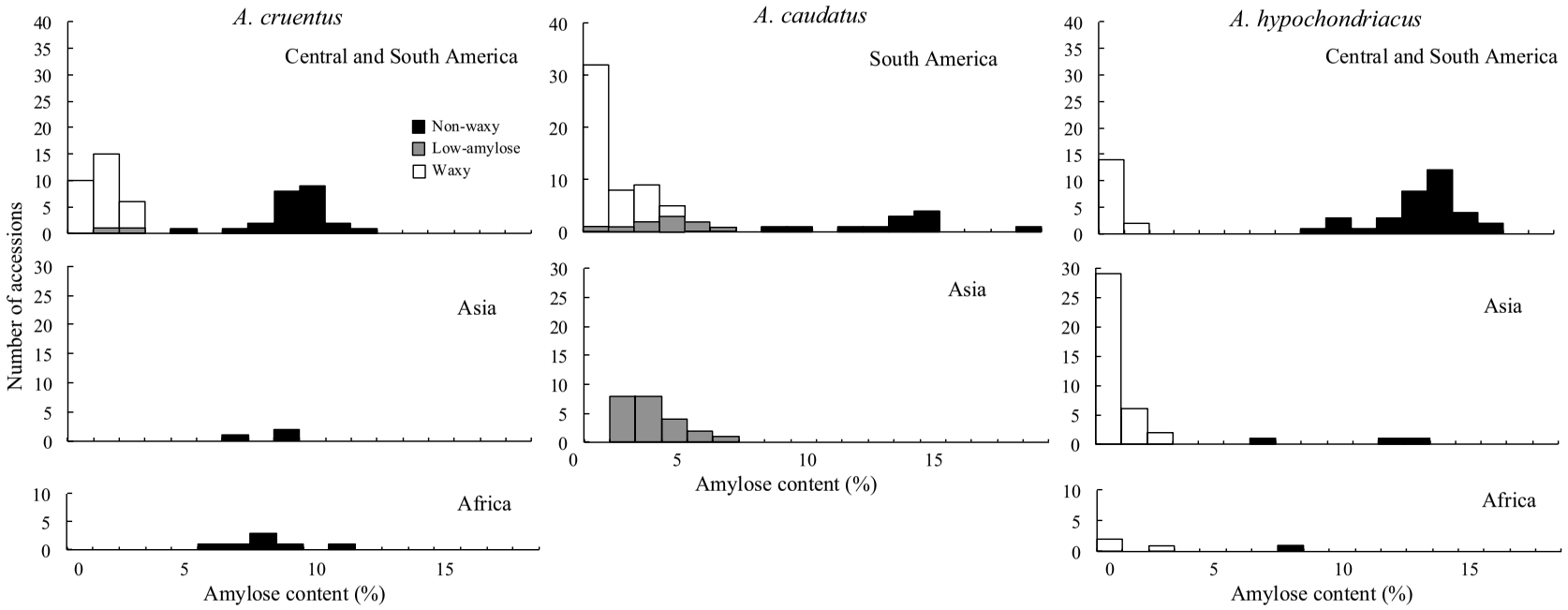


Fig. 2. Geographical variation of contentand waxy starch type in three grain amaranth species.

non-waxy type. In Central America, 30 of 33 accessions from Mexico were of the waxy type, while only one of 15 accession from Guatemala was of the waxy type. In *A. caudatus*, all of the perisperm types were found in accessions from Peru and Bolivia in South America, while, with the exception of two waxy accessions from India, all of the accessions from Asia were of the low amylose type. In *A.hypochondriacus*, while the non-waxy type was dominant among the accessions from Mexco, all of the accessions from Asia and Africa were of the waxy type, except for three non-waxy accessions from Nepal and Nigeria.

2-4. Discussion

2-4-1. Variation in amylose content and perisperm type

Broad variation of amylose content in perisperm of the three species was revealed in this study using materials collected from different parts of the world. The amylose content in grain amaranths has been reported previously in a few samples (Sugimoto *et al*. 1981; Tomita *et al*. 1981; Konishi *et al*. 1985; Inouchi *et al*. 1999). These studies can summarize that *A. cruentus* has amylose contents ranging from 0 to 28%, *A. caudatus* from 5.0 to 6.9% and *A. hypochondoriacus* from 0 to 21.8%, respectively. In case of *A. caudatus*, nallow variation of amylose contents and only low amylose type were reported (Tomita *et al*. 1982). Consequently, this is thus the first report to show the existence of a wider variation in amylose content and that both waxy and non-waxy types exist.

Compared to previous studies, the relatively low amylose contents observed in this study can be explained by the reason that whole grain powder was used for analysis and that amylose contents were calculated using a calibration curve of starch granules (Table 2). In grain amaranths, generally, the starch content of whole grains is approximately 50 to 60% (Saunders and Becker 1984). Then, assuming that it is 50%, the amylose content in starch granule becomes just double. Based on this assumption, the range of amylose contents was 0 to 35.4%, corroborating previous report, which is the almost same range of rice (0 to 30%, Katsuta *et al*. 1989) and foxtail millet (0 to 25.1% Takei *et al*. 1989; Nakayama *et al*. 1998).

2-4-2. Geographical distribution

The obvious gap in the geographical distribution of amylose contents and perisperm types was revealed between the New World and the Old World in three species. The accessions from Central and South America exhibited a wide variation in amylose contents and all perisperm types. Conversely accessions from Asia and Africa showed a limited variation in amylose contents and a particular type of perisperm types; *A. cruentus* has only non-waxy type, *A. caudatus* has only low-amylose type with exception of two accessions from India, and *A. hypochondriacus* has only waxy type with exception of three accessions from Nepal and Nigeria, respectively. Interestingly, different perisperm starch types were introduced from the area of origin to the Old World, i.e. non-waxy type of *A. cruentus*, low amylose type of *A. caudatus* and waxy type of *A. hypochondoriacus*, respectively. These results suggest that the geographical gap was developed by the bottle neck, the limited population were disseminated from Central and South America to Asia and Africa, and also the frequency of dissemination might be few.

Indeed, all of the low amylose type *A. caudatus* accessions in Asia have the same morphological traits. The inflorescence is drooping and red color, and the seeds have a translucent perisperm with red rim (embryo part) (Fig.1-D), while most accessions from South America have elect or semi-elect inflorescence and variation of seed color (data not shown). The drooping inflorescence type is commonly referred as 'Love-Lies-Bleeding' etc. in Europe, which is grown as an ornamental plant. According to Sauer (1950), this variety may have been introduced into Asia from South America via Europe. In addition, it is assumed that this variety was selected for in Europe based on the characteristic of inflorescence and not because it has a low amylose content. However, after the introduction into Asia, especially to the Indian subcontinent, *A. caudatus* has been accepted as one of the grain crops and not as an ornamental.

In *A. cruentus*, accessions from Asia and Africa had only dark brown or black seed coats and morphologically similar inflorescences. In addition, the seed size of them also was significantly smaller than that seeds with a pale-yellow seed coat (data not shown). This indicates that these accessions have been used as vegetables in the regions.

In this case, the starch type of perisperm cannot distinguish directly by the appearance of perisperm into non-waxy and waxy type because of the colored seed coat (Fig. 1A). In other words, perisperm starch type is not important for vegetable use. In Central America, the proportions of non-waxy and waxy accessions from Guatemala and Mexico were completely different, even though the countries are adjacent. In Guatemala, 14 of 15 accessions were of the non-waxy type and 10 of the accessions had the same dark brown or black seed coat as the accessions from Asia and Africa. In Guatemala, amaranth is typically called '*bledo*' in Spanish and is generally used as a relict vegetable (Sauer 1967). Conversely, in Mexico, 30 of 33 accessions were waxy type and amaranth is a staple grain crop. It is consider that the difference of main usage in between Guatemala and Mexico caused such results.

A. hypochondriacus showed a different result from *A. cruentus*. Almost all accessions from Asia and Africa had a waxy type and whitish yellow seed coat. In the foothills of Himalayan range in Asia (e.g. Nepal, India, Pakistan and Bhutan), *A. hypochondriacus* is widely cultivated as a staple grain crop (Sauer 1967; Sakamoto 1993). Similarly, an investigation of more than 200 Nepalese accessions of *A. hypochondoriacus* by the author revealed that only three non-waxy accessions were found (K. Nemoto unpublished). However, in a survey conducted in Kathmandu and Langtang valley in Nepal in 1975, Sakamoto (1982) reported that of 16 samples three were of the non-waxy type and one was a mixture of both types. These findings indicate that, compared to today, the proportion of non-waxy type may have been higher than several decades ago.

2-4-3. Origin of the waxy and low-waxy type

In this study, amylose content and perisperm starch type were investigated using a large number of accessions from different regions of the three species. The results revealed that the diversity of the perisperm starch type in the New World was cleared. Sakamoto (1983) assumed that the waxy type of *A. hipochondriacus* had already differentiated from the non-waxy type in the New World in pre-Columbian times. This assumption was based on the fact both the waxy and non-waxy type had

been found in Central America where the species had been domesticated. The results of our study not only support the hypothesis of Sakamoto, but also indicate that the waxy and low amylose perisperm types of *A. cruentus* and *A. caudatus* were developed in the New World. Maize, which had been domesticated in the New World as well as grain amaranths, also has a waxy endosperm type . Waxy mutant was first discovered in China (Collins 1909) and were particularly widely distributed in East Asia after dissemination to the Old World. However, unlike grain amaranth, waxy mutant has been selected in the Old World (Sakamoto 1982), although some were found in North America and Argentina (Bregger 1928; Mangeksdorf 1924). Grain amaranths are, therefore, the only crop, which were established the waxy type by human selection in the New World.

However, why was the waxy type selected for in grain amaranth? The waxy endosperm type in cereal crops was selected for the preference of sticker trait (Sakamoto 1989). A specific food preference for waxy endosperm starch exists in East Asia where developed the waxy form of seven cereal crops, such as rice, foxtail millet, common millet, Job's tears, sorghum, barley and maize (Sasaki 1982; Sakamoto 1989). In this area, the sticker trait of waxy endosperm starch has played an important role for its particular food culture. Generally, waxy grains have own recipes using the sticky trait and are clearly cooked to distinguish the non-waxy grains. On the other hand, in the case of *A. hypochondoriacus*, Sakamoto (1983, 1997) considered that the waxy periperm type may have been established as a result of a preference for a lighter seed coat (See Fig 2-B, C). Because popped seeds are used mostly for food preparation and no clear difference can be found in the utilization of the non-waxy type and the waxy type in grain amaranths. Indeed, except for the Chepang, a minor ethnic group in Nepal, utilization of the stickier waxy starch compared to the non-waxy starch has never been reported (manuscript in preparation). Consequently, as proposed by Sakamoto (1989), the selection pressures to establish waxy perisperm starch type of grain amaranths is quite different from that for the waxy endosperm types of cereal crops in East Asia where there is a well developed preference for sticky food.

The low amylose types of both *A. cruentus* and *A. caudatus* were also

considered to be the New World in origin. However, the perisperm of the low amylose and non-waxy type are both translucent and they cannot be distinguished each other by the appearance. Consequently, the reason underling the selection for the low amylose type instead of the non-waxy type cannot to be attribute to a particular preference for sticky food or for a lighter seed coat. This suggests that the low amylose type was not selected consciously from non-waxy type at all. Indeed, the accessions of low amylose type in *A. caudatus* were localized at Asia. This was deduced that small samples introduced into Europe as an ornamental plant, which have a long and drooping inflorescence as mentioned before, were of the low amylose type by chance and then disseminated to Asia.

2-5. References

Bregger, F. G. 1928. Waxy endosperm in Argentin maize. J. Heredity **19**: 111.

Collins, G.N. 1909. A new type of Indian corn from China. USDA, Plant Indust. Bur. Bull. **161**: 1-30.

Inouchi N., K. Nishi, S. Tanaka, M. Asai, Y. Kawase, Y. Hata, Y. Konishi, S. Yue, and H. Fuwa 1999. Characterization of amaranth and quinoa starch. J. Appl. Glycosci. **46**: 233-240.

Katsuta M, T. Nagamine, S. Oka, K. Okuno, and Y. Egawa 1989. Genetic variations and geographic distribution on amylose content of Asian rice cultivars. Jpn. J. Breed. **39** (suppl. 2): 258-259. (in Japanese)

MacMasters M. M., P. D. Baird, M. M. Holzapfel, and C. E. Rist 1955. Preparation starch from *Amaranth cruentus* seed. Economic Bot. **9**: 300-302.

Mangelsdorf P.C. 1924. *Waxy* endosperm in New England maize. Science, **60**: 222-223.

Nakayama H. M. Afzal, and K. Okuno 1998. Intraspecific differentiation and geographical distribution of *Wx* alleles for low amylose content in endosperm of foxtail millet, *Setaria italica* (L.) P. Beauw. Euphytica **102**: 289-293.

Okuno K. and S. Sakaguchi 1982. Inheritance of starch characteristics in perisperm of *Amaranthus hypochondriacus*. J. Heredtiy **73**: 467

Sakamoto S. 1982. *Waxy* endosperm and perisperm of cereals and grain amaranth and their geographical distributions (in Japanese with English summary). J. Japan Soc. Starch Sci. **29**: 41-55.

Sakamoto S. 1989. Mochi no Bunkashi (Glutinous-endosperm and – perisperm Starch Food Culture). Chuoukouronsha (Tokyo) p.175. (in Japanese).

Sakamoto S. 1997. Origin and ethnobotany of glutinous perisperm starch found in a species of grain amaranths, *Amaranth hypochondriacus* L. Intercultural Studies, Ryukoku University **1**: 124-133.

Sasaki K. 1982. Shoyoujurin Bunka no Michi: Bhutan, Unnan kara Nihon e (Shouyoujurin Culture Comprex; From Bhutan, Yunnan to Japan). Nihon Housou Shuppan Kyoukai (Tokyo) p.253. (in Japanese).

Saunders R. and R. Becker 1984. *Amaranthus*: a potential food and feed resource. Adv. Cereal. Sci. Technol. **6**: 357-396.

Sauer J. D. 1950. The grain amaranths: A survey of their history and classification. Ann. Mo. Bot. Gard. **37**: 561-632.

Saure J. D. 1967. The grain amaranths and their relatives: A revised Taxonomic and geographic survey. Ann. Mo. Bot. Gard. **54**: 103-137.

Sauer J. D. 1969. Identity of archaeological grain amaranths from the valley of Tehuacan, Puebla, Mexico. American Antiquity **34**: 80-81.

Sauer, J. D. 1976. Grain amaranths. In Simmonds NW (ed.) Evolution of crop plants, Longman (NewYork) pp. 4-7.

Sugimoto Y., K. Yamada, S. Sakamoto, and H. Fuwa 1981. Some properties of normal and waxy-type starches of *Amaranthus hypochondriacus* L. Stärke **33**: 112-116.

Takei E, H. Taira, and S. Sakamoto 1989. Geographical variation of amylose content in foxtail millet (*Setalia italica* P. Beauw.). Japan J. Breed. **39** (Suppl. 2): 260-261. (in Japanese)

Taki M. 1959. Studies on the determination of amylose of starches by means of paper chromatography. J. Agr. Chem. Soc. Japan **33**: 245-249. (in Japanese)

Tomita Y, Y. Sugimoto, S. Sakamoto, and H. Fuwa 1980. Some properties of starchs of grain amaranths and several millets. J. Nutr. Sci. Vitaminol **27**: 471-84.

Wolf M. J., M. M. MacMasters, and C. E. Rist 1950. Some characteristics of the starches three South American seeds used for food. Cereal Chemistry **27**: 219-222.

Chapter 3

Genetic Diversity of Grain Amaranths (*Amaranthus* spp.) revealed by SDS-PAGE of Seed Proteins

3-1. Introduction

Grain amaranths (*Amaranthu*s spp.) consist three cultivated species that is, *A. caudatus* L.*, A. cruentus* L., and *A. hypochondriacus* L. In ancient times, grain amaranths were basic and staple crops in the Americas, where these originated, before the arrival of the Spanish Conquistadors (Sauer 1950). From the archaeological records, the history of their cultivation has been considered to be about 6,000 years (Sauer 1969). Thus, it is expected that these grain species have accumulated genetic variation in their gene pool throughout this long period.

To evaluate genetic variation is important for genetic improvement of the crop. Local and exotic germplasm can be used as a source of genetic variation. At the same time, a large scale of analysis is required for understanding the variability. Seed protein electrophoresis is one of the useful tools to evaluate genetic diversity due to its physiological stability and trait relatively less affected by the selection and environment (Ladizinsky and Hymowitz 1979). In a number of crops, therefore, the seed protein profiles by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been utilized not only to assess inter and/or intra- specific genetic diversity (Tomooka *et al*. 1992; Rogl and Javornik 1996; Isemura *et al*. 2001; Karihaloo *et al*. 2002; Anu and Peter 2003), but also to reveal phylogenetic relationship (Singh *et al*. 1991; Jha and Ohri 1996; Mirali *et al*. 2007), to identify varieties (Ferguson and Grabe 1986; Hussain *et al*. 1988) and so on.

Several studies on seed protein electrophoresis in Genus *Amaranthus* have been reported (Gudu and Gupta 1988; Gorinstein and Moshe 1991; Braba de la Rosa *et al*. 1992; Segura-Niet *et al*. 1992; Sammour *et al*. 1993; Zheleznov *et al*. 1997; Drzewiecki 2001; Juan *et al*. 2007). However, the previous studies used a small number of accessions that were not a complete representation of the variability of grain

amaranths.

The objectives of the present study were to assess intra and inter-specific variation of grain amaranths and to investigate the geographical distribution of their electrophoretic band type.

3-2. Materials and Methods

3-2-1. Plant materials

A total of 312 accessions of grain amaranths, 117 of *A. hypochondriacus*, 77 of *A. cruentus*, and 117 of *A. caudatus*, collected from various parts of the world including Central and South America, Asia, and Africa were used in this study (Table 1). They were supplied by the USDA-ARS National Plant Germplasm Amaranth Collection (USA) and Shinshu University (Japan). Some strains were mixed with different seed coat color and/or appearance of the perisperm. In that case, seeds were separated and analyzed as a different strain, respectively.

3-2-2. Seed protein extraction

Total seed protein was extracted from 5mg of seed flour with 200μl of 0.05M Tris-HCl buffer (pH 6.8) containing 1% SDS, 1% 2-mercaptoethanol and 20% glycerol. Prior to centrifugation at 10,000 rpm for 15min, the extract was incubated in boiling water for three minutes. The extracted protein was recovered as clear supernatant and 40μl of 0.02% Bromophenol Blue was then added to the extract.

3-2-3. Electrophoresis

SDS-Polyacrylamid gel electrophoresis (SDS-PAGE) of the extracted proteins was analyzed on 10.83% polyacrylamide mini-slab gel according to the methods of Laemmli (1970). The electrophoresis was performed at 100V constant. Low Molecular Weight Calibration Kit (Pharmacia Biotech) was used as the molecular weight standards. All gels were stained with Coomassie Brilliant blue G-250 and destained by diffusion in 10% CH_3OH-30% CH_3COOH-water.

Table 1. Origin, seed protein types and number of strains in three grain amaranth species

Origin	No. of strains	Seed protein types									
		I	II	III	IV	V	VI	VII	I+II	II+IV	III+V
A. hypochondriacus											
Central and South America											
Mexico	50	1	22	-	2	-	1	16	8	-	-
United States	2	-	2	-	-	-	-	-	-	-	-
Puerto Rico	1	-	1	-	-	-	-	-	-	-	-
Brasil	1	-	1	-	-	-	-	-	-	-	-
Chile	1	-	1	-	-	-	-	-	-	-	-
Asia											
Nepal	28	-	28	-	-	-	-	-	-	-	-
Pakistan	7	-	7	-	-	-	-	-	-	-	-
India	14	-	14	-	-	-	-	-	-	-	-
China	5	-	5	-	-	-	-	-	-	-	-
Bhutan	3	-	3	-	-	-	-	-	-	-	-
Afganistan	1	-	1	-	-	-	-	-	-	-	-
Africa											
Nigeria	2	-	2	-	-	-	-	-	-	-	-
Uganda	1	-	1	-	-	-	-	-	-	-	-
Zambia	1	-	1	-	-	-	-	-	-	-	-
Total	117	1	89	0	2	0	1	16	8	0	0
A. cruentus											
Central and South America											
Mexico	34	-	1	-	31	-	-	-	-	2	-
Guatemala	17	-	-	-	17	-	-	-	-	-	-
United States	8	1	-	-	7	-	-	-	-	-	-
Peru	2	-	-	-	2	-	-	-	-	-	-
Asia											
India	5	-	4	-	1	-	-	-	-	-	-
Africa											
Nigeria	3	-	3	-	-	-	-	-	-	-	-
Gana	3	-	3	-	-	-	-	-	-	-	-
Zambia	3	-	3	-	-	-	-	-	-	-	-
Zimbabwe	1	-	1	-	-	-	-	-	-	-	-
Zaire	1	-	1	-	-	-	-	-	-	-	-
Total	77	1	16	0	58	0	0	0	0	2	0
A. caudatus											
South America											
Peru	43	-	-	42	-	1	-	-	-	-	-
Bolovia	36	-	-	36	-	-	-	-	-	-	-
Argentina	9	-	-	1	-	5	-	-	-	-	3
Ecuador	1	-	-	1	-	-	-	-	-	-	-
Guatemala	3	-	-	3	-	-	-	-	-	-	-
Asia											
Nepal	17	-	-	-	-	17	-	-	-	-	-
India	3	-	-	-	-	3	-	-	-	-	-
Pakistan	3	-	-	-	-	3	-	-	-	-	-
Bhutan	2	-	-	-	-	2	-	-	-	-	-
Total	117	0	0	83	0	31	0	0	0	0	3
Grand total	312	2	56	83	60	31	1	16	8	2	3

3-3. Results

Variation of the SDS-PAGE electrophoretic pattern of total seed proteins was observed in the range from 54 to 68 kDa (Fig. 1). Of these polymorphic bands, the band appeared at 67 kDa was excluded in the present variation analysis because this is *Wx* protein band, which is a granule-bound starch synthase and associated with amylose synthesis (Konishi *et al*. 1985; Braba de la Rosa *et al*. 1992; Park *et al*. 2010). Total seven different bands designated as I (ca. 54 kDA), II (57 kDA), III (59 kDA), IV (61 kDA), V (63 kDA), VI (66 kDA) and VII (ca. 68 kDA), respectively, and their combination of banding pattern, namely I+II, II+IV and III+V were detected and used in the study (Fig. 2). Of the 312 strains examined, type I contained 2 strains, type II 56, type III 83, type IV 60, type V 31, type VI 1, type VII 16, type I+II 8, type II+IV 2, and type III+V 2 (Fig. 2). As for the 13 strains with multiple bands, taking into consideration of the possibility of contamination of the different type of seeds in material, re-examination was carried out using single seeds in each. As a result, it was confirmed not due to the contamination in all strains (data not shown).

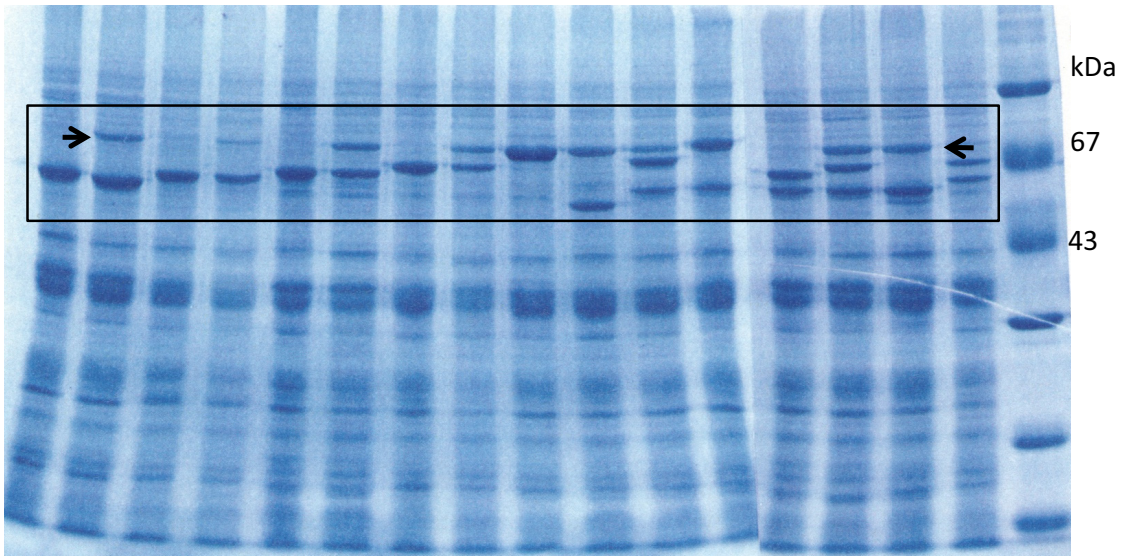


Fig. 1. The SDS-PAGE electrophoregram of total seed protein in grain amaranths. Arrows indicate the position of *Wx* protein band.

Then, for inter and intra-specific variation, six different types were recognized in *A. hypochondriacus* (Table 1). Type II was the most and type VI was the second most frequent. Each type accounted for 76% and 14%, respectively. Most of the banding pattern of type I appeared in combination with type II. The geographical distribution of them differed remarkably. While all types were observed in Mexican strains, only type II was found in the strains from Asia, Africa and even the other central and South American countries. *A. cruentus* had four different types. Type IV showed the highest frequency and type II was the next accounted for 75% and 21% respectively. Type II+IV was found only in Mexico. The geographical distribution of both type II and IV were clearly different. In central and South American countries, type IV was detected without a strain from India. On the other hand, type II was observed in Asian and African countries without a strain from Mexico. Type I with a very low frequency was detected from the strain in the USA. *A. caudatus* contained three different types, that is type III, V and III+V accounted for 71%, 27% and 2%, respectively. The distribution of type III was restricted in South American countries. In contrast, type V was found 6 strains from Peru and Argentina in South America, whereas, all strains from Asian countries showed this type. Type III+V was found only in Argentina. Type I, II and IV were a common to *A. hypochondriacus* and *A. cruentus*. *A. hypochondriacus* had two species-specific band, that is type VI and VII, but *A. cruentus* had no species-specific band. In contrast, *A. caudatus* did not have common band of *A. hypochondriacus* and *A. cruentus*, and had only two species-specific bands.

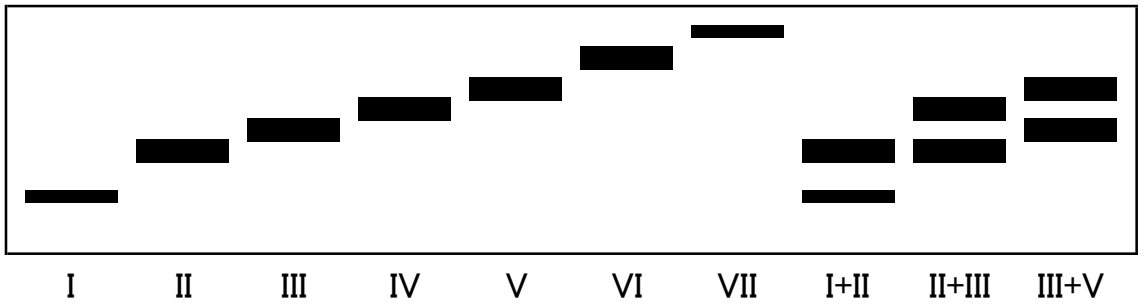


Fig. 2. Schematic illustration of 7 types and 3 their combination types.

3-4. Discussion

In the present study, intra- and interspecific variations of seed storage proteins in grain amaranths were clarified by using 312 strains collected from diverse regions. Total ten different banding patterns were recognized (Fig. 2). Of these, three had multiple bands that seem to the result of introgression in both types. Very similar banding patterns detected in the range from 54 to 68 kDa were also found in previous reports (Gudu and Gupta 1988, Braba de la Rosa *et al*. 1992; Segura-Nieto *et al*. 1992; Sammour *et al*. 1993; Drzewiecki 2001; Juan *et al*. 2007). Higher interspecific variation was observed in *A. hypochondriacus*, which has 6 different types, in comparison with both *A. cruentus* (4 types) and *A. caudatus* (3 types). A similar tendency was found in the variation of *GBSSI* (also called *Waxy*) gene, which encodes granule bound starch synthase I (GBSSI) (Park *et al*. 2012). These indicate that genetic diversity of *A. hypochondriacus* is relatively higher than other species. Interestingly, all the variation found in *A. cruentu*s, that is the type I, II and IV, was included in the variation of *A. hypochondriacus*, which was observed in strains from Mexico. This suggests that genetic relationship between *A. hypochondriacus* and *A. cruentus* is much closer than *A. caudatus.* Although the phylogenetic relationship among these three species has still been controversial, similar relationship has been reported by isozyme and RAPD analysis (Chan and Sun 1997), and Low-Cot DNA sequences (Sun *et al*. 1999). Further analysis on overlapped bands in between *A. hypochondriacus* and *A. cruentus* will be necessary, for example by the analysis of particular proteins such as globulin, albumin and prolamin and/or two-dimensional electrophoresis.

In terms of intraspecific variation, geographical distribution of the types differed greatly. In *A. hypochondriacus*, the variation was found only in Mexico where the species domesticated (Sauer 1967). The result indicates that very restricted gene pool, type II in this case, disseminated other region and the event was considered to be few. In case of *A. cruentus*, apart from the place of origin in Mexico and Guatemala (Sauer 1967), particular type (type II), which was detected with only one strain in Mexico, was spread to Asia and Africa. The type of morphological character in inflorescence was clearly distinguished from that of type IV (data not shown). And also

the seeds of all strains in type II was colored black and significantly smaller than typical seeds for grain use including type IV. Although type II in *A. cruentus* overlapped with that in *A. hypochondriacus*, the morphology including inflorescence was completely different. However, the reason is unclear why only that type of strains exists in Asia and Africa. *A. caudatus* showed similar tendency with *A. cruentus*. Only type V observed in South America, where is the area of origin (Sauer 1967) with very low frequency, was detected in Asia. As described in Nemoto *et al*. (submitted), the morphology of inflorescence is different both type III and V each other. Inflorescence of type II elects, whereas that of type V is drooping. Three strains with type III+V found in Argentina were considered to be a species that was once identified as *A. edulis* because of its semi drooping inflorescence.

Interspecific variation of seed storage proteins in grain amaranths revealed in the study, which has from three to six different banding patterns. From the results, the polymorphic single bands can utilize to identify the species, to classify into some groups roughly and to check the hybridization among different types as genetic markers. Especially, type III and V can use for the identification of *A. caudatus* because it has only species-specific bands. For the purpose of genetic improvement, we have started interspecific cross breeding work. In the work, the dwarf seedlings that seem to be a hybrid lethal phenomenon were observed in the cross between type III and V in *A. caudatus*. The same result was reported in the cross between certain strains in the species (Coons 1992). In order to overcome this situation, a strain of type III+V expects to use as a bridge material of genetic exchange between type III and V.

3-5. References

Anu A. and K. V. Peter 2003. Analysis of seed protein of 29 lines of *Capsicum annum* L. by plyacrylamid gel electrophoresis. Genetic Resources and Crop Evolution **50**: 239-243.

Braba de la Rosa, A. P., J. Gueguen, O. Paredes-Lopez and G. Viroben 1992. Fractionation procedures, electrophoretic characterization, and amino acid composition of amaranth seed proteins. J. Agric. Food Chem. **40**:931-936.

Coons, M. P. Relationships of *Amaranthus caudatus*. Economic Botany **36**(2): 129-146.

Chan K. F. and M. Sun 1997. Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. Theor Appl Genet **95**: 865-873.

Drzewiecki J. 2001. Similarities and differences between *Amaranthus* species and cultivers und estimation of outcrossing rate on the basis of electrophoretic separations of urea-soluble seed proteins. Euphytica **119**: 279-287.

Ferguson J. M. and D. F. Grabe 1986. Identification of cultivars of *Stylosanthes capitata* Vog. by polyacrylamide gel electrophoresis of seed proteins. Crop Science **26**: 170-176.

Gorinstein, S. and R. Moshe 1991. Evaluation of four *Amaranthus* species through protein electrophoretic patterns and their amino acid composition. J Agric. Food Chem. **39**: 851-854.

Gudu S. and V. K. Gupta 1988. Electrophoresis as an aid for identification of various species and cultivars of grain amaranths. Acta Horticulturae **218**: 230-238.

Hussain, A. H. Ramirez, W. Bushuk and W. M. Roca 1988. Identification of cultivars of *Stylosanthes capitata* Vog. by polyacrylamide gel electrophoresis of seed proteins. Euphytica **37**: 117-119.

Isemura, T., C. Noda, S. Mori, M. Yamashita, H. Nakanishi, M. Inoue and O Kamijima 2001. Genetic Variation and Geographical Distribution of Azuki Bean (*Vigna angularis*) Landraces Based on the Electrophoregram of Seed Storage Proteins. Breeding Science **51**: 225-230.

Jha, S. S. and D. Ohri 1996. Phylogenetic relationships of *Cajanus cajan* (L.) Millsp. (pigeonpea) and its wild relatives based onseed protein profiles. Gnenetic Resources and Crop Evolution **43**: 275-281.

Juan R., J. Pastor, M. Alaiz and J. Vioque 2007. Electrophoretic characterization of *Amaranthus* L. seed proteins and its systematics implications. Botanical Journal of the Linnean Society **155**: 57-63.

Karihaloo, J. L., M. Kaur and S. Singh 2002. Seed protein diversity in *Solanum melongena* L. and its wild and weedy relatives. Genetic Resources and Crop

Evolution **49**: 533-539.

Konishi Y., H. Nojima, K. Okuno, M. Asaoka and H. Fuwa 1985. Characterization of starch granules from waxy, nonwaxy, and hybrid Seeds of *Amaranthus hypochondriacus* L. Agric. Biol. Chem. **49**(7): 1965-1971.

Ladizinsky G. and T. Hymowitz 1979. Seed protein electrophoresis in taxonomic and evolutionary studies. Theor. Appl. Genet. **54**: 145-151.

Laemmli U. K. 1970. Cleavage of structure proteins during assembly of the head of bacteriophage T4. Nature **22**:680-685.

Mirali, N. S. El-Khouri and F. Rizq 2007. Genetic diversity and relationships in some *Vicia* species as determined by SDS-PAGE of seed proteins. Biologia Plantarum **51**(4): 660-666.

Park Y. J, K. Nemoto, T. Nishikawa, K. Matsushima, M. Minami and M. Kawase 2010. Waxy strains of three amaranth grains raised by different mutation in the coding region. Mol. Breeding **25**: 623-635.

Park Y. J., T. Nishikawa, N. Tomooka and K Nemoto 2012. The molecular basis of mutations at the Waxy locus from *Amaranthus caudatus* L.: evolution of the waxy phenotype in three species of grain amaranth. Mol. Breeding **30**: 511-520.

Rogl, S. and B. Javornik 1996. Seed protein variation for identification of common buckwheat (*Fagopyrum esclentum* Moench) cultivars. Euphytica **87**: 111-117.

Sammour H. R., M. A. Hammoud and S. A. Abd Alla 1993. Electrophoretic variations in *Amanrahtus*. Bot. Bull. Acad. Sin. **34**: 37-42.

Sauer J. D. 1950. The grain amaranths: a survey of their history and classification. Ann. Mo. Bot. Gard. **37**: 561-619.

Sauer J. D. 1967. The grain amaranths and their relatives: A revised taxonomic and geographic survey. Ann. Mo. Bot. Gard. **52**(2): 103-137.

Sauer J. D. 1969. Identity of archaeological grain amaranths from the valley of Tehuacan. Puebla, Mexico, American Antiquity **34**: 80-81.

Segura-Nieto, M., N. Vazquez-Sanchez, H. Rubio-Velazquez, L.R. Olguin-Martinez, C. Rodiguez-Nester and L. Herrera-Estrella 1992. Characterisation of amaranth (*Amaranthus hypochondriacus* L.) seed proteins. J. Agric. Food Chem. **40**: 1553–

1560.

Singh, A. K., S. Sivaramakrishunan, M. H. Mengesha and C. D. Ramaiah Phlogenetic relations in section Arachis based on seed protein profile. Theor. Appl. Genet. **82**:593-597.

Sun M., H. Chen and F. C. Leung 1999. Low-cot DNA sequences for fingerprinting analyasis of germplasm diversity and relationships in *Amaranthus*. Theor. Appl. Genet. **99**: 464-472.

Tomooka, N., C. Lairungreang, P. Nakeeraks, Y. Egawa and C. Thavarasook 1997. Center of genetic diversity and dissemination pathways in mung bean deduced from seed protein electrophoresis. Theor. Appl. Genet. **83**: 289-293.

Zheleznov A. V., Solonenko L. P. and N. B. Zheleznova 1997. Seed proteins of the wild and the cultivated *Amaranthus* species. Euphytica **97**: 177-182.

Chapter 4

Phylogenetic study between grain amaranths and their wild relatives revealed by AFLP analysis

4-1. Introduction

Grain amaranths (*Amaranthus* spp.) are ancient crops originated in the Americas as important food staple for thousands of years (Brenner *et al*. 2000). These contain three cultivated grain species and distributed geographically separate locations, that is, *A. hyphocondiracus* L. is in northwestern and central Mexico, *A. cruentus* L. is in southern Mexico and Guatemala and *A. caudatus* L. is in the Andes of South America, respectively (Sauer 1950, 1967). Sauer (1967) demonstrated that three of putative wild progenitors, *A. hybridus*, *A. quitensis* and *A. powellii*, were to be involved deeply in the domestication process of the grain species. And he claimed the two hypotheses regarding the evolutionary relationships between grain amaranths and their wild relatives through his precise taxonomic and geographic survey (Sauer 1967, 1976). One is the single progenitor hypotheses that *A. cruentus* domesticated from *A. hybridus* and then *A. hypochondriacus* and *A. caudatus* evolved secondarily by repeated crossing of *A. cruentus* with two other wild species, *A. powellii* in the north and *A. quitensis* in the south. The other is the independent domestication hypothesis that the grain species was domesticated independently from different wild species, *A. hypochondriacus* from *A. powellii*, *A. cruentus* from *A. hybridus* and *A. caudatus* from *A. quitensis*.

Sauer's two hypotheses are always in a starting point when verifying the relationships. To date, many studies on the phylogenetic relationships of the grain amaranths have been reported (*e.g.* Pal and Khoshoo 1972; Hauptli and Jain 1984; Gudu and Gupta 1988; Gupta and Gudu 1991; Transue *et al*. 1994; Lanoue *et al*. 1996; Chan and Sun 1997; Sun *et al*. 1999; Xu and Sun 2001; Mandal and Das 2002; Mallory *et al*. 2008; Maughan *et al*. 2011; Das 2012; Kietlinski *et al*. 2013; Park *et al*. 2014). However the relationships among them are still controversial because the results between the previous studies are incoherent. So that, the origins of the grain amaranths

are not clearly elucidated and have not been studied in great detail (Kietlinski *et al*. 2013). Therefore, accumulation of further research is required.

Amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995) has the potential to resolve phylogenetic relationships, particularly among closely related species, or intraspecific level (Despres et al. 2003; Meudt and Clarke 2007). In grain amaranths, there are some previous studies using this approach to identify the accessions that are ambiguous taxonomically at the basic morphological level (Costea *et al*. 2006) and to analyze the phylogenetic relationships (Xu and Sun 2001).

The purpose of this study is to examine phylogenetic relationships between grain amaranths and their wild relatives, and to investigate intraspecific variation of grain amaranths based on the analysis of AFLP markers using accessions collected from various region of the world. And also we discuss the relationships added our results of seed protein profile of them by SDS-PAGE (Nemoto *et al*. submitted to TAD and Nemoto, unpublished data).

4-2. Materials and methods

4-2-1. Plant materials

A total 96 accessions from six species of the genus *Amaranthus* were used in AFLP analysis (Table 1). The cultivated species were represented by 23 *A. caudatus* (CA), 26 *A. cruentus* (CR) and 36 *A. hypochondriacus* (HP) accessions. The wild species were represented by 7 *A. hybridus* (HB), 3 *A. powellii* (PO) and 4 *A. quitensis* (QU) accessions. All accessions were obtained from the collection at the United States Department of Agriculture (USDA), USA, and the Shinshu University, Japan. Information of seed protein type was also noted in Table 1.

4-2-2. DNA extractions

Yung leaf tissues from 2-week-old seedlings were harvested for DNA isolation and then these samples were immediately frozen in liquid nitrogen and stored at -80℃until use. Genomic DNA was extracted from young leaves using the CTAB method (Murray and Thompson 1980) with some modifications.

Table 1 Materials used in this experimetns

Species	No.	Accession No.	Origin	Seed protein type	Species	No.	Accession No.	Origin	Seed protein type
A. caudatus	ca1	Ames 15176	Argentina	III	*A. hypochondriacus*	hp1	Ames 5436	Mexico	II+VII
	ca2	Ames 15177	Argentina	III		hp2	Ames 5467	Mexico	I
	ca3	Ames 15179	Argentina	III+V		hp3	PI 511731	Mexico	VI
	ca4	PI 481607	Bhutan	V		hp4	PI 604580	Mexico	II
	ca5	PI 490604	Bolivia	III		hp5	Ames 5132	Mexico	V
	ca6	PI 490606	Bolivia	III		hp8	PI 477917	Mexico	II
	ca7	PI 490607	Bolivia	III		hp9	PI 604576	Mexico	II
	ca8	PI 568139	Bolivia	III		hp10	PI 490755	Mexico	II
	ca9	PI 568143	Borivia	III		hp11	PI 604560	Mexico	II
	ca10	PI 568153	Borivia	III		hp12	PI 604794	Mexico	II
	ca11	PI 166107	India	V		hp13	Ames 5158	Puerto Rico	II
	ca12	PI 175039	India	V		hp14	Ames 5689	Brazil	II
	ca13	Ames 10176	Pakistan	V		hp15	Ames 5355	Chile	II
	ca14	PI 490546	Peru	III		hp16	Ames 21766	China	II
	ca15	PI 490614	Peru	III		hp17	PI 542595	China	II
	ca16	PI 490621	Peru	III		hp18	PI 590991	China	II
	ca17	PI 490626	Peru	III		hp19	PI 337611	Uganda	II
	ca18	PI 490639	Peru	III		hp20	Ames 1972	Nigeria	II
	ca19	PI 511683	Peru	III		hp21	Ames 1975	Nigeria	II
	ca20	PI 511693	Peru	III		hp22	PI 558499	United States	II
	ca21	PI 511705	Peru	III		hp23	PI 274279	India	II
	ca22	PI 511753	Peru	V		hp24	85-10-10-3-15	India	II
	ca23	IB 85-3291	Nepal	V		hp25	Almora	India	II
A. cruentus	cr1	Ames 22000	Guatemala	IV		hp26	85-10-27-3-5	India	II
	cr2	Ames 22004	Guatemala	IV		hp27	Ac#00406	Sri Lanka	II
	cr3	Ames 5676	Guatemala	IV		hp28	PI 540446	Pakistan	II
	cr4	PI 511715	Guatemala	IV		hp29	Ames 5609	Afghanistan	II
	cr5	PI 511718	Guatemala	IV		ho30	N-203	Bhutan	II
	cr6	Ames 5165	United States	I		hp31	Ames 5660	Zambia	II
	cr7	Ames 5318	United States	IV		hp32	N-107	Nepal	-
	cr8	Ames 5677	United States	IV		hp33	N-125	Nepal	-
	cr9	Ames 5480	Mexico	IV		hp34	SU87-871478	Nepal	-
	cr10	Ames 15189	Mexico	IV		hp35	PI 511721	Mexico	II+V
	cr11	PI 451710	Mexico	IV		hp36	PI 604564	Mexico	II
	cr12	PI 490662	Mexico	II+IV	*A. hybridus*	hb1	Ames 2026	United States	VI
	cr14	PI 511726	Mexico	II+IV		hb2	Ames 5684	United States	II+VI
	cr15	PI 576481	Mexico	IV		hb3	Ames 21188	South Africa	IV
	cr16	PI 604558	Mexico	IV		hb4	Ames 22002	Guatemala	IV
	cr17	PI 511713	Peru	IV		hb5	PI 500249	Zambia	IV
	cr18	Ames 1977	India	II		hb6	PI 490754	Mexico	IV
	cr19	Ames 2037	India	II		hb7	PI 604671	United States	IV
	cr20	PI 566897	India	IV	*A. quitensis*	qu1	Ames 23372	Brazil	V
	cr21	PI 576448	Nigeria	II		qu3	PI 511736	Bolivia	III
	cr22	Ames 1968	Ghana	II		qu4	PI 511745	Ecuador	III
	cr23	Ames 5369	Zaire	II		qu5	PI 568154	Bolivia	III
	cr24	PI 494774	Zambia	II	*A. powellii*	po1	PI 538796	Russia	New Type
	cr25	PI 604569	Mexico	IV		po2	PI 572257	Rwanda	New Type
	cr26	PI 490657	United States	IV		po3	PI 572261	Germany	New Type

4-2-3. AFLP analysis

Genomic DNA (20 ng) was restriction digested using the enzyme combination *Eco*RI and *Mse*I, and then ligated to *Eco*RI and *Mse*I adapters: *Eco*RI adaptor, 5'-CTCGTAGACTGCGTACC-3', 3'-CATCTGACGCATGGTTTAA-5'; *Mse*I adaptor, 5'-GACGATGAGTCCTGAG-3', 3'-TACTCAGGACTCAT-5' (AFLP Core Reagent Kit, Life Technologies, Carlsbad, CA). Pre-selective amplifications were performed with an

*Eco*RI+0 (5'-GACTGCGTACCAATTC-3') and *Mse*I+0 (5'-GATGAGTCCTGAGTAA-3') primer combination during 20 PCR cycles each at 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. After pre-selective amplifications, selective amplifications were performed using five primer combinations (ACA/CAT, ACT/CAG, AAC/CAT, ACC/CTC and AAG/CAT) in the AFLP Selective Amplification Start-Up Module (Applied Biosystems, Tokyo, Japan). The polymerase chain reaction (PCR) conducted in 10 μl volumes containing 1 μl of pre-selective DNA, 1 μl of 10×Ex Taq buffer, 0.8 μl of 2.5 mM dNTP mixture, 1 μl of both primers, and 0.25 μl of EX Taq polymerase. We used the following touch-down PCR conditions: one cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, followed by 13 cycles in which the annealing temperature was progressively lowered by 0.7°C and finally 33 cycles at 94°C for 30 s, followed by 56°C for 30 s and 72°C for 60 s. The PCR products of selective amplification were electrophoresed in 6% polyacrylamide gel for 2 hours at 65W. The gels were stained with Vistra Green I (GE Healthcare) washed thoroughly double distilled water and photographed by using FluorChem (Alpha Innotech Corporation, San Leandro, CA).

4-2-4. Data analysis

AFLP raw data were scored for the presence (1) or absence (0) of homologous bands for all samples. Estimates of similarity among all genotypes were calculated according to Dice's index of similarity using version 8.0 of the program SPSS (SPSS Inc., 1989-2002, Chicago, Illinois, U.S.A.). Phylogenetic analysis was performed using the neighbor joining (NJ) method (Saitou and Nei 1987) with PHYLIP ver. 3.6b (Felsenstein 1995).

4-3. Results

4-3-1. AFLP analysis

AFLP analysis revealed a large number of distinct, scorable fragments per primer pair (Table 2). In the 96 accessions of the 6 species from *Amaranthus* studied, a total of 298 fragments were amplified from four primer combinations:

*Eco*RI-ACA/*Mse*I-CAT, 89 fragments; *Eco*RI-ACT/*Mse*I-CAG, 64 fragments; *Eco*RI-AAC/*Mse*I-CAT, 71 fragments; *Eco*RI-ACC/*Mse*I-CTC, 74 fragments. Among these, 276 were polymorphic (92.6%). The proportion of polymorphic within species is shown as follows: *A.caudatus*, 63%; *A.cruentus*, 75%; *A.hypochondriacus*, 89%; *A.hybridus*, 68%; *A.quitensis*, 75% and *A.powellii*, 85% (Table 3).

Table 2 Polymorphism rate of primer pairs

Primer combination	Total bands	Polymorphic bands	Polymorphism rate (%)
*Eco*RI+3-ACA/*Mse*I+3-CAT	89	81	91
*Eco*RI+3-ACT/*Mse*I+3-CAG	64	57	89.1
*Eco*RI+3-AAC/*Mse*I+3-CAT	71	68	95.8
*Eco*RI+3-ACC/*Mse*I+3-CTC	74	70	94.6
Total	298	276	92.6

Table 3 Percentage of polymorphism in each species

Species	No. of accessions	Total No. of bands	No. of plymorhic bands	Polymorphism (%)
A. caudatus	23	170	108	63.5
A. cruentus	25	205	155	75.6
A. hypochondriacus	34	240	215	89.6
A. hybridus	7	196	134	68.4
A. powellii	3	210	179	85.2
A. quitensis	4	215	162	75.3

4-3-2. Phylogenetic analysis

The relationships among the species analyzed are shown in Fig. 1. Three major Group A, B and C were evident in the phylogenetic analysis of the AFLP matrix as demonstrated by the NJ tree. The first upper clade, Group A comprised *A. caudatus* and *A. quitensis*; the second clade, Group B comprised *A. cruentus*, *A.hypochondriacus* and *A.hybridus*; the third Group C comprised only *A.powellii*. All accessions were clustered together well for each species, except that accessions of *A. hybridus* were split into two clades in Group B.

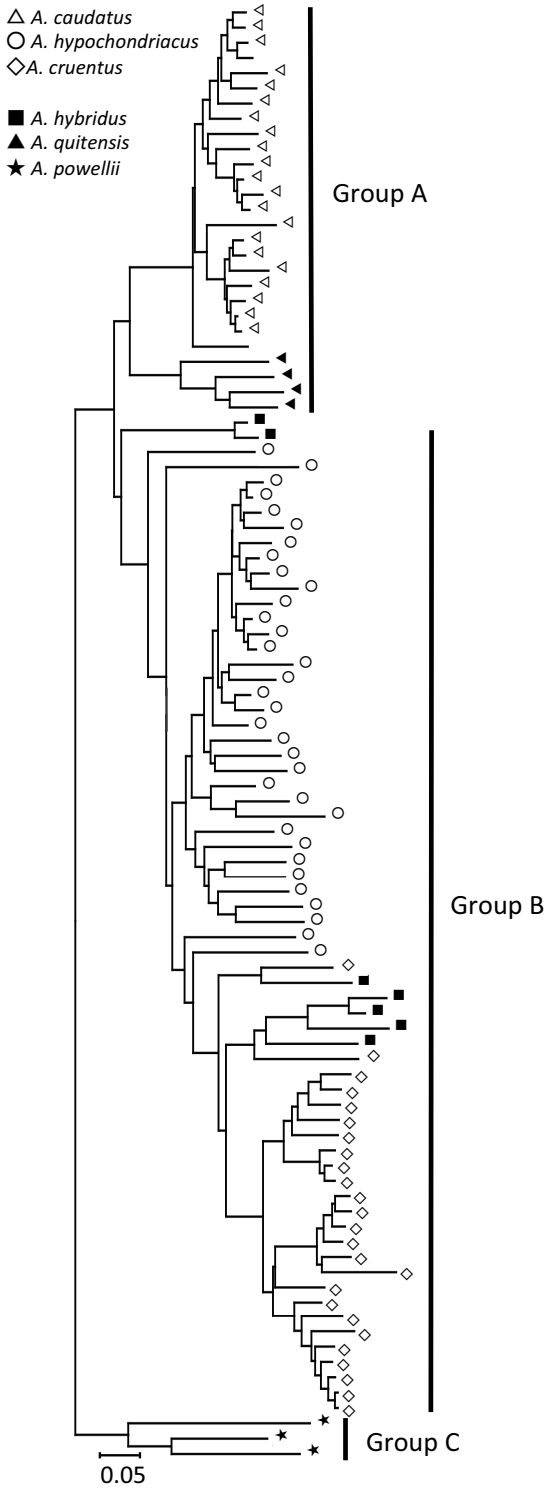


Fig. 1 A NJ phenogram based on AFLP markers obtained with four primer pairs. The scale is the measure of genetic similarity calculated according to Nei and Li (1979).

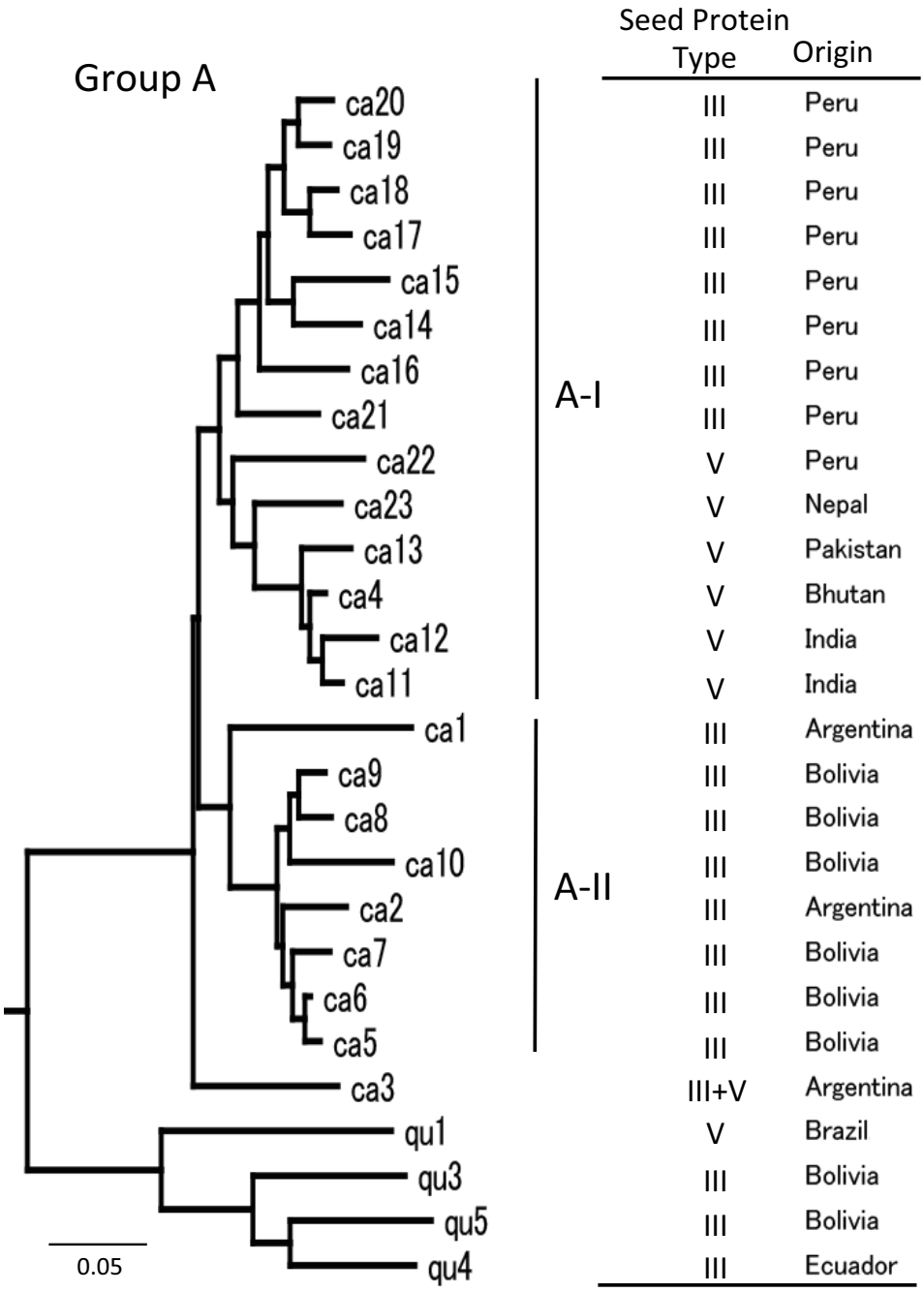
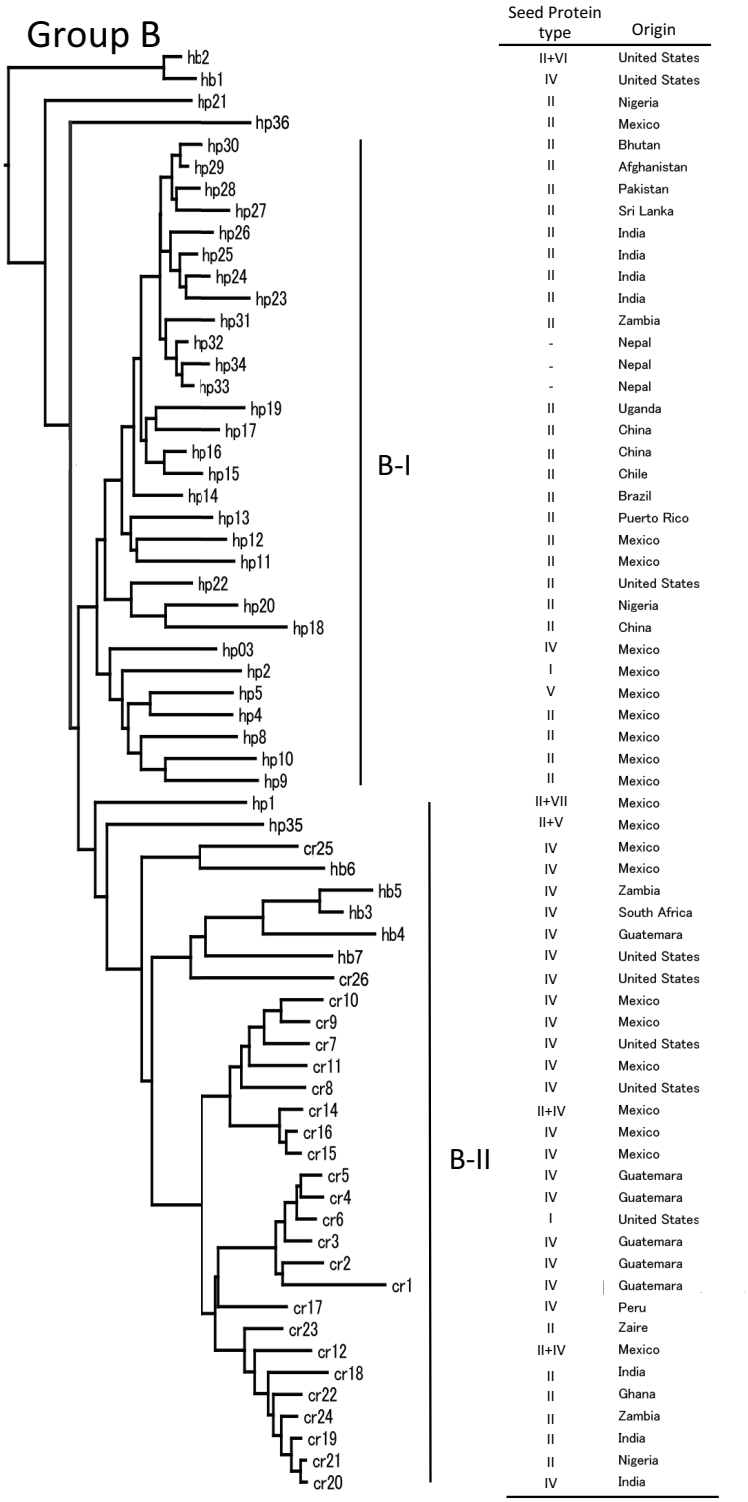


Fig. 2 A NJ phenogram of Group A composed of *A. caudatus* (ca) and *A. quitensis* (qu). The scale is the measure of genetic similarity calculated according to Nei and Li (1979).The seed protein type and origin are indicated on the right side of the panel.



Seed Protein type	Origin
II+VI	United States
IV	United States
II	Nigeria
II	Mexico
II	Bhutan
II	Afghanistan
II	Pakistan
II	Sri Lanka
II	India
II	India
II	India
II	India
II	Zambia
-	Nepal
-	Nepal
-	Nepal
II	Uganda
II	China
II	China
II	Chile
II	Brazil
II	Puerto Rico
II	Mexico
II	Mexico
II	United States
II	Nigeria
II	China
IV	Mexico
I	Mexico
V	Mexico
II	Mexico
II	Mexico
II	Mexico
II	Mexico
II+VII	Mexico
II+V	Mexico
IV	Mexico
IV	Mexico
IV	Zambia
IV	South Africa
IV	Guatemara
IV	United States
IV	United States
IV	Mexico
IV	Mexico
IV	United States
IV	Mexico
IV	United States
II+IV	Mexico
IV	Mexico
IV	Mexico
IV	Guatemara
IV	Guatemara
I	United States
IV	Guatemara
IV	Guatemara
IV	Guatemara
IV	Peru
II	Zaire
II+IV	Mexico
II	India
II	Ghana
II	Zambia
II	India
II	Nigeria
IV	India

Fig. 3 A NJ phenogram of Group B composed of *A. hybridus* (hy), *A. hypochondriacus* (hp) and *A. cruentus* (cr). The scale is the measure of genetic similarity calculated according to Nei and Li (1979). The seed protein type and origin are indicated on the right side of the panel.

Group A was clearly divided into each species of *A. caudatus* and *A. quitensis* (See Fig. 1 and Fig. 2). Accessions of *A. caudatus* were further subdivided into two subgroups, designated as A-I and A-II (Fig. 2). The subgroup A-I comprised of 7 accessions from Peru and 5 from Asia. In subgroup A-II, 6 accessions were from 4 Bolivia and 2 from Argentina.

In Group B, two accessions of *A. hybridus* (hb1 and hb2) were configured the outermost of the clade (Fig.1 and Fig. 3). The group was also including sister to two subgroups, B-I and B-II (Fig. 3). The subgroup B-I was consisting of *A. hypochondriacus* only. Subgroup B-I was further consisted of two clade. One is primarily consisted of Asian and African accessions, and the other was consisted of only Mexican accessions. The subgroup B-II mainly consisted of *A. cruentus,* and some accessions of *A. hybridus* and *A. hypochondriacus* were placed on the outside of the clade. Two taxa of *A. hybridus*, which split in the group, showed a different morphology (Fig. 4). The accessions configured the outermost (hb1 and hb2) were a 'weed type' (Fig. 4-A) that has large branches extending outside with inflorescence in the bottom. It looks a typical pigweed. On the other hand, another accessions (hb3, hb4, hb5, hb6 and hb7) were a 'cultivated-like type' (Fig. 4-B) that has a tall main stem more than 2 meter with many small branches but dose not have big inflorescence like grain species.

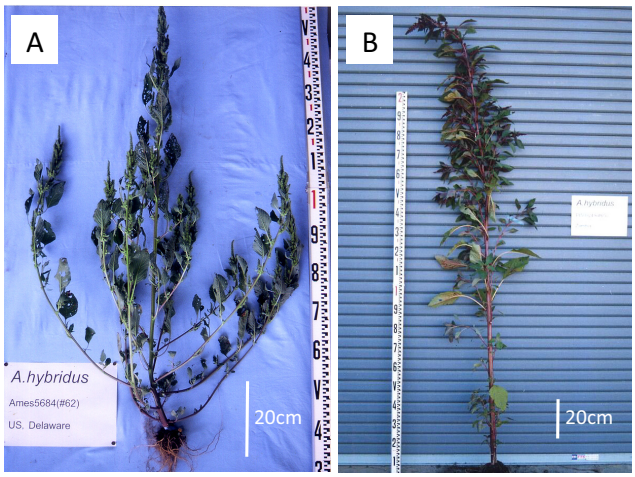


Fig. 4 Different plant type found in accessions of *A. hybridus* used in the study. A: weed type (hb2), B: cultivated-like type (hb5).

4-4. Discussion

4-4-1. Intra- and interspecific variation of grain amaranths

In the present study, high levels of genetic variation were detected and verified within and among grain amaranths species. The rate of polymorphism averaging 92.6% was high as same as the result of Xu and Sun (2001). Within species, however, AFLP polymorphism had been kept at a relatively higher level ranging from 63.5 to 89.6% unlike the result of Xu and Sun (2001). *A. hypochondriacus* showed the highest intraspecific variation among the grain species. Higher level of variation in the species in comparison with *A. cruentus* and *A. caudatus* was obtained in our studies such as the *granule-bound starch synthase I* (*GBSSI*) gene (Park *et al*. 2012), the *starch branching enzyme* (*SBE*) gene (Park *et al*. 2014), the amylose content in seed storage starch (Nemoto *et al*. submitted to TAD) and the seed protein profiles by SDS-PAGE (Nemoto *et al*. submitted to TAD). This tendency may have indicated the possibility that the period after domestication of *A. hypochondriacus* is longer than other two species, considering the accumulation of genetic variation in population/species increases basically with the passage of time.

Accessions in clades within grains species tends to come together geographically and same seed protein type (Fig. 2 and 3). In Subgroup A-I of *A. caudatus* (see Fig. 2), in particular, Asian accessions having the seed protein type V were taken together with accessions from Peru having the Type III in the same clade. This suggests that the variation of Type V found in all Accessions in Asia derives from Peruvian population. From the fact that *A. quitensis* had only Type III and V as same of *A. caudatus*, it clearly indicated that both species are closely related.

Two morphologically different types found in *A. hybridus* (Fig. 4) had a common seed protein type each other, the ‘weed type’ had Type VI and the ‘cultivated-like type’ had Type IV, respectively. In *A. hypochondriacus*, only one accession (hb18) with the same type of the ‘weed type’ was found in Mexico. On the other hand, the Type IV that the ‘cultivated-like type’ has was s major seed protein type in *A. cruentus*. Kietlinski *et al*. (2013) showed similar result by SSR analysis and proposed the possibility of existence of two widespread morphologically different taxa.

4-4-2. Phylogenetic relationships

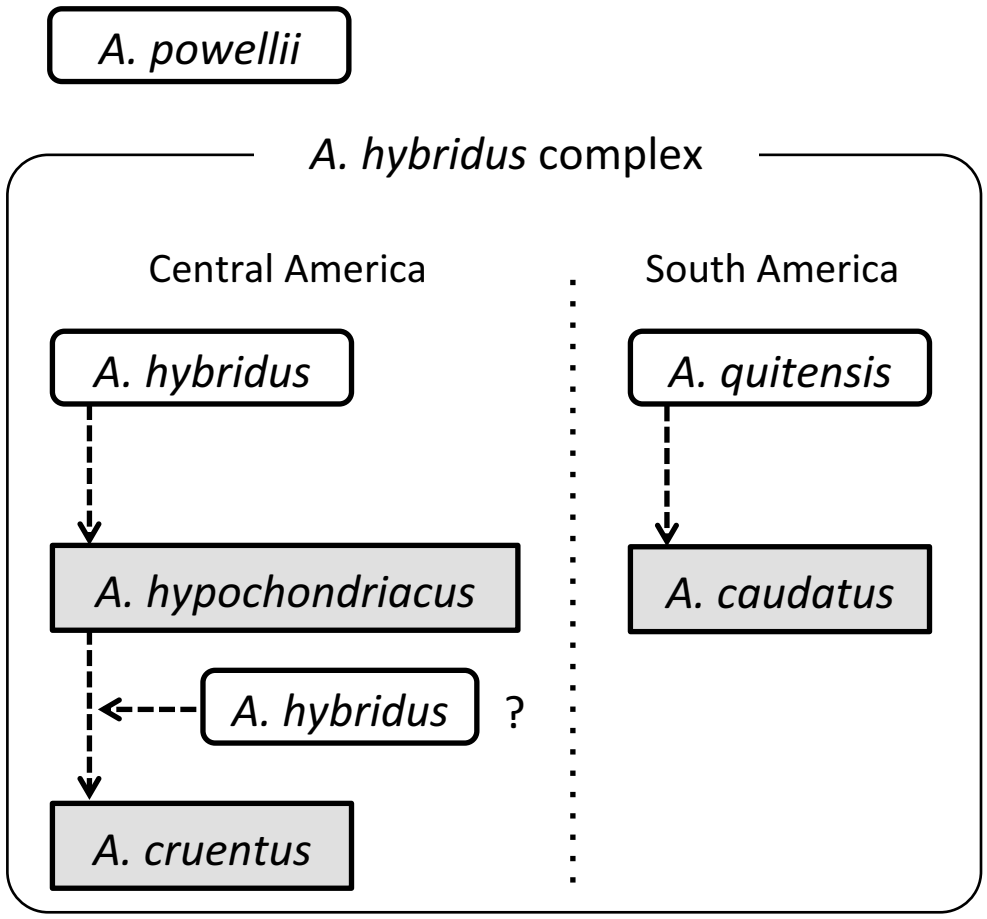


Fig. 5 Schematic flow of evolutionary origin of three species of grain amaranths based on the results of AFLP and seed protein analysis. The *arrows* indicate the direction of domestication.

4-5. References

Murray, M. G. and W. F. Thompson 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8**: 4321-4325.

Felsenstein J. 1995. PHYLIP version 3.6b. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA 98195 USA.

Meudt, M. H. and A. C. Clarke 2007. Almost forgotten or latest practice? AFLP

applications, analysed and advances. Trends in Plant Science **12**(3): 106-117.

Despres, L., L. Gielly, B. Redoutet and P. Taberlet 2003. Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Molecular Phylogenetics and Evolution **27**: 185-196.

Brenner, D. M., D. D. Baltensperger, P. A. Kulakow, J. W. Lejmann, R. L. Myers, M. M. Slabbert and B. B. Sleugh 2000. Genetic resources and breeding of *Amaranthus*. Plant Breed. Rev. **19**: 227-285.

Chan, K. F. and M. Sun 1997. Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. Theor. Appl. Gnenet. **95**(5-6): 865-873.

Costea, M., D. M. Brenner, F. J. Tardif, Y. E. Tan and M. Sun 2006. Delimitation of *Amaranthus cruentus* L. and *Amaranthus caudatus* L. using micromorphology and AFLP analysis: An application in germplasm identification. Genet. Resour. Crop Evol. **53**(8): 1625-1633.

Costea, M., A. Sandres and G. Waines 2001. Preliminary results toward a revition of the *Amaranthus hybridus* species complex (Amaranthaceae). SIDA 19: 931-974.

Gudu, S. and V. K. Gupta 1988. Electrophoresis as an aid in the identification of variation species and cultivers of grain amaranths. Acta Hortic. **218**: 231-238.

Gupta V. K. and S/ Gudu 1991. Interspecific hybrids and possible phylogenetic relations in grain amaranths. Euphytica **52**: 33-38.

Kietlinski, K. D., F. Jimenez, E. N. Jellen, P. J. Maughan. S. M. Smith and D. B. Pratt 2013. Relationships between the weedy Amaranthus hybridus (Amaranthaceae) and the grain amaranths. Crop Sci. **54**: 220-228.

Lanoue K, P. Wolf, S. Browning, E. Hood 1996. Phylogenetic analysis of restriction-site variation in wild and cultivated *Amaranthus* species (Amaranthaceae). Theor. Appl. Genet. **93**:722–732.

Mallory, M., R. Hall, A. R. McNabb, D. B. Pratt, E. Jellen and P. Maughan 2008. Development and characterization of microsatellite markers for the grain amaranths (*Amaranthus* spp. L.). Crop Sci. **48**: 1098-1106.

Mandal N. and P. K. Das 2002. Intra-and interspecific genetic diversity in grain *Amaranthus* using random amplified polymorphic DNA markers. Plant Tissue culture **12**: 49-56.

Maughan, P. J., S. M. Smith, D. J. Fairbanks and E. N. Jellen 2011. Development, characterization, and linkage mapping of single nucleotide polymorphisms in the grain amaranths (*Amaranthus* sp.). Plant Gen. **4**: 1-10.

Saitou, T. and M. Nei 1897. The neighbor-joining methods: a new method of reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406-425.

Sauer, J. D. 1950. The grain amaranths: A survey of their history and classification. Ann. Mo. Bot. Gard. **37**: 561-631.

Sauer, J. D. 1967. The grain amaranths and their relatives: A revised taxonomic and geographic survey. Ann. Mo. Bot. Gard. **54**(2): 103-137.

Sauer, J. D. 1976. Grain amaranths. In: Evolution of Crop plants. (Simmonds, N. W. ed.) Longman (London) pp. 4-7.

Sun, M., H. Chen and G. C. Leung 1999. Low-Cot DNA sequences for relationships in *Amaranthus*. Theor. Appl. Genet. **99**: 464-472.

Transue, D. K., D. J. Fairbanks, L. R. Robison and W. R. Andersen 1994. Species identification by RAPD analysis of grain amaranth genetic resources. Crop Sci. 35(3): 1385-1389.

Xu, F. and M. Sun 2001. Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (Amaranthus; Amaranthaceae) using internal transcribed spacer, amplified fragment length polymorphism, and double-primer fluorescent intersimple sequence repeat markers. Mol. Phylogenet. Evol. **21**(3): 372-387.

Park, Y. J.,

Park, Y. J., T. Nishikawa, K. Matsushima, M. Minami, N. Tomooka and K. Nemoto 2014. Molecular characterization and genetic diversity of the s*tarch branching enzyme* (*SBE*) gene from Amaranthus: the evolutionary origin of grain amaranths. Mol. Breeding **34**: 1975-1985.

Das S. 2012. Systematics and taxonomic delimitation of vegetable, grain and weed

amaranths: a morphological and biochemical approach. Genet. Resour. Crop Evol. **59**:289–303.

Hauptli H, and S. Jain 1984. Allozyme variation and evolutionary relationships of grain amaranths (*Amaranthus* spp.). Theor. Appl. Genet. **69**:153–165.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. **12**: 4407-4414.

Chapter 5

General discussion

Amylose content is a major factor affecting grain quality, and it is controlled almost exclusively by GBSSI. Therefore, improvements to the quality of amaranth grain, including economic and functional improvements, may depend on understanding the action and effects of genes encoding this enzyme. To date, however, there is no basic information on the *GBSSI* gene in grain amaranths. The aim of the present study was to examine the *GBSSI* gene and the molecular basis of its mutants. First, the gene encoding amaranth GBSSI protein (and its mutants) was isolated and characterized (Chapter 2 and 3). Then, to trace the evolutionary and geographical origins of the waxy phenotype, the genetic diversity of the *Waxy* locus was examined (Chapter 4 and 5). Finally, the expression pattern of the *GBSSI* gene was determined by analyzing expression in seeds at different developmental stages and in storage and non-storage tissues (Chapter 5). The molecular structure of the amaranth *GBSSI* gene, as determined in the present research, is discussed in detail.

6.1 Characterization of GBSSI in grain amaranth

As described in Chapter 2, a full-length cDNA clone encoding GBSSI was first isolated and characterized from the perisperm of *A. cruentus* L. Segregation of amylose content in the F_2 population suggested that this trait was controlled by a single gene, *GBSSI*, in *A. cruentus* (Table 2.3), as is the case in *A. hypochondriacus* (Okuno and Sakaguchi 1982). Analyses of the structure of the gene revealed that the amaranth *GBSSI* gene is similar to that of the nonwaxy type characterized in other species such as maize, rice, foxtail millet, potato, and sweet potato (Klosgen et al. 1986; van der Leij et al. 1991; Okagaki 1992; Fukunaga et al. 2002; Kimura et al. 2000). In those five species, the *GBSSI* gene contains 14 exons, with 13 exons (exons 2–14) contributing to the coding sequence. In this study, the 5′ UTR, including exon 1 and intron 1, was not detected. However, the amaranth *GBSSI* gene contained 13 exons (coding sequence)

and this structure was similar to that of the *GBSSI* gene in the other five plant species. Further research is required to clarify the role of the 5' UTR, and the implications of its absence from *Amaranthus*.

The mature protein shares 70.2–75.3% amino acid sequence identity with GBSSI of dicots and approximately 64.0–67.8% identity with those of monocots. It contains the conserved motif KTGGL that is found in other GBSSI proteins. This motif has been implicated as the active site in glycogen synthase (Furukawa et al. 1990, 1993). Sequence analyses predicted that the GBSSI of amaranth has a transit peptide of 77 amino acids including FIR↓S, which is a different cleavage site from that in the GBSSIs of other dicot species. In addition, there are more polymorphic sites in the GBSSIs of dicots, including that of amaranth, than in GBSSIs of monocots. Some sites of the amaranth GBSSI, for example, the transit peptide including the cleavage site, have amino acids that differed from those of GBSSIs in other dicots. These results were well reflected in the relationships among species in the phylogenetic tree (Fig. 2.6). This tree indicated that amaranth was sister to the subgroup consisting of the rest of the dicots, which cluster more closely than with monocots. Thus, the significant difference in the transit peptide of amaranth GBSSI provided an additional character that supported the relationships suggested by phylogenetic analyses of GBSSI sequences. The phylogenetic analysis using *SSSII* (Soluble Starch Synthase II) genes of various plants showed a similar result (Yan et al. 2009). That tree also indicated that amaranth is sister to the subgroup consisting of the rest of the dicots, which cluster separately from the monocots.

6.2 Genetic distances among three grain amaranth species based on *Waxy* sequences

The sequences of the *Waxy* gene were also used to determine the genetic distances among the three *Amaranth* species. The resulting kimura 2-parameter distance revealed the longest or shortest pair-wise genetic distances. The shortest genetic distance (0.00124) was between *A. caudatus* (*Wx-ca*) and *A. cruentus* (*Wx-cr*), and the second shortest genetic distance (0.00218) was between *A. cruentus* (*Wx-cr*) and *A.*

hypochondriacus (*Wx-hy*). The longest genetic distance (0.00342) was between *A. cruentus* (*Wx-ca*) and *A. hypochondriacus* (*Wx-hy*). Similar studies on genetic variations in grain amaranths have been carried out using restriction-site variations of chloroplast nuclear DNA (Lanoue et al. 1996), isozymes and random amplified polymorphic DNAs (RAPDs) (Chan and Sun 1997), amplified fragment length polymorphisms (AFLPs) and inter simple sequence repeats (ISSRs) (Xu and Sun 2001), and micromorphology and AFLPs (Costea et al. 2006). According to these reports, *A. caudatus* and *A. cruentus* had the closest relationship among the three grain amaranths. Therefore, information about the sequences of the *Waxy* gene may provide useful data to explain the inter- and/or intra-specific relationships in amaranths, although more accessions should be included to verify such relationships.

6.3 Waxy strains of three amaranth grains arose from different mutations in the coding region

GBSSI mutations essentially eliminate or reduce amylose content from the starch through the disrupted expression or decreased functioning of the *Waxy* gene. Naturally occurring *waxy* mutants have been found in several plant species, and the mechanisms underlying such mutations have been well characterized in rice, maize, wheat, barley, and foxtail millet (Fedoroff et al. 1983; Wessler and Varagona 1985; Wang et al. 1995; Cai et al. 1998; Hirano et al. 1998; Vrinten et al. 1999; Domon et al. 2002; Fukunaga et al. 2002).

The structural variations between *Waxy* and *waxy* alleles from the coding region of each of the three *Amaranth* species were examined (Chapter 3). The results revealed a frame-shift mutation in the *Waxy* gene in *A. caudatus* and a nonsense mutation in that of *A. cruentus* and *A. hypochondriacus*. These mutations in the coding region of the *Waxy* genes were responsible for the change in perisperm starch, resulting in the waxy phenotypes (Fig. 3.3). This is the first report of differences in the coding sequences that can explain the absence of a functional waxy protein in each of the three *waxy* alleles. Screening for mutation points in a large gene pool of the *Waxy* locus was also conducted. These analyses were carried out using PCR-RFLP, AS-PCR, and direct

sequence analysis of genes from various waxy phenotypes of the three amaranth grains (Chapters 3 and 4). The results clearly showed that all waxy strains have the same mutation point, resulting in the conversion of *Waxy* into *waxy* alleles. This demonstrates that a single nucleotide polymorphism altered the regulation of the *Waxy* gene during the domestication of grain amaranths. Usually, a nonsense mutation causes the termination of translation and then translation of the mRNA transcribed from this mutant gene stops prematurely. A similar nonsense mutation was detected in Japonica-type rice (Isshiki et al. 2001) and waxy barley (Domon et al. 2002).

6.4 Origin and evolution of waxy phenotype in grain amaranth

Knowledge of the genetic variations in *Amaranth* is important for studies on crop evolution. In Chapters 4 and 5, polymorphisms in a large gene pool of the *Waxy* locus from samples of grain amaranths collected from diverse regions were investigated to clarify the origins and evolution of this crop plant. The phylogenetic analysis of *A. hypochondriacus*, which was based on *Waxy* variations, indicated that three types of waxy sequence occurred separately and independently in certain domesticated regions in Mexico (Fig. 4.4). In all of the waxy strains of *A. hypochondriacus*, the *GBSSI* gene showed the same mutation point, at which the *Waxy* gene was converted into the waxy phenotype as a result of a G-A polymorphism. A similar result was found when the *Waxy* locus of the other two species, *A. caudatus* and *A. cruentus,* was examined. In *A. cruentus*, comparison of the *GBSSI* coding sequence among 37 strains revealed an extremely high level of sequence conservation, and a single nucleotide change (a G-T polymorphism) between the sequences of the nonwaxy (Type I) and waxy (Type II) phenotypes (Chapter 5). In the case of *A. caudatus*, the *Waxy* locus showed a very high level of sequence conservation (data not shown). In particular, the insertion of a single nucleotide into the coding region of the *A. caudatus Waxy* gene resulted in a complete loss of gene function, which was correlated with the waxy phenotype. These results indicate that the waxy mutations in the three grain amaranths probably occurred independently in their domesticated regions with waxy varieties derived from a single mutational event (i.e., a frame shift or base substitution). Thus, the present results

demonstrate that the current distribution of waxy strains reflects a simple evolutionary process and a monophyletic origin.

Grain amaranths originated in the New World, and are an ancient crop that was already under cultivation 5000–7000 years ago (Sauer 1967). According to Sauer (1950, 1967), the three grains amaranths were independently domesticated from wild species in three regions: *A. caudatus* from *A. quitensis* in South America, *A. cruentus* from *A. hybridus* in Central America, and *A. hypochondriacus* from *A. powellii* in Mexico. In addition, both non-waxy and waxy strains of grain amaranths are cultivated to this day in the New World (Okuno and Sakaguchi 1982). Therefore, the mutation in grain amaranths perisperm from the non-waxy phenotype to the waxy phenotype would have naturally occurred during the cultivation history of these crops, although the age and history of the mutation events remain unknown.

Crop evolution is a symbiotic process involving plants and humans, and it has led to drastic changes in the genetic control of plant traits (Kawase et al. 2005). The waxy types of most crops were directly selected and propagated because of a specific preference for sticky food. Interestingly, Sakamoto (1997) suggested that the waxy type of grain amaranth was indirectly established as a result of a preference for a whiter seed coat color in the early domestication history of this crop. Therefore, the evolutionary history of the waxy phenotype of grain amaranth may differ from that of other waxy-starch crops, which were directly selected by peoples who favored sticky foods in East Asia (Sakamoto 1997).

6.5 Comprehensive expression analyses of *GBSSI* in grain amaranth

The expression pattern of the *GBSSI* gene in the perisperm of grain amaranth was investigated as a first step toward understanding the relationship between transcription of this gene and protein (amylose) accumulation. Previous observations indicated that the transcript and protein of GBSSI in several cereals was mainly localized in storage organs (Hirose and Terao. 2004; Dian et al. 2005; Ohdan et al 2005). This gene was designated as a ‘later expresser’ because abundance of its transcripts increased from approximately 5 or 7 days after flowering (Ohdan et al. 2005).

In the present study, the abundance of *GBSSI* transcripts was higher at the middle developmental stage when starch accumulated most rapidly in the perisperm. The transcript abundance was relatively lower at the very stage of perisperm development, before there was significant starch production in the perisperm (Fig. 5.3 and 5.4). This finding is consistent with those of previous studies on the expression patterns of the *GBSSI* gene in rice seeds (Dry et al., 1992; Hirose and Terao, 2004). However, the expression pattern of the *GBSSI* gene differed among various other tissues. It was expressed in all tested non-storage tissues (leaf, stem, petiole, and root) and its expression tended to increase during leaf development (Fig 5.5). Thus, the amaranth *GBSSI* gene is expressed in both storage and non-storage organs.

In the present study, the starch from pericarp tissue of amaranth stained blue-black and contained amylose at the initial developmental stage (Fig. 5.3). This observation suggests that there could be a second *GBSS* gene in the pericarp. In fact, in many species (for example pea, barley, and rice) *GBSSI* is responsible for synthesis of amylose in the seed, and *GBSSII* is responsible for amylose synthesis in other organs (Denyer et al. 1997; Nakamura et al. 1998; Edwards et al. 2002; Hirose and Terao 2004; Ohdan et al. 2005). In potato and *Arabidopsis*, however, only one *GBSSI* gene is responsible for amylose synthesis throughout the plant (Edwards et al. 2002). It would be interesting and straightforward to determine whether there are two *GBSS* genes in grain amaranth. The isoforms of *GBSSII* in grain amaranth will be investigated in future studies to obtain a complete picture of amylose biosynthesis in this crop plant.

Based on the results of the present study, the process of starch granule (including amylose) accumulation in amaranth storage tissue can be divided into four stages: (1) synthesis of starch granules in the pericarp during the initial developmental stage; (2) disappearance of starch granules and termination of starch synthesis in the pericarp, and initiation of starch synthesis in the perisperm at an early developmental stage; (3) synthesis of starch granules leading to a rapid increase in the perisperm during the middle and mid-late developmental stages; and (4) a decline in synthesis of starch granules at the late developmental stage.

6.6 Future directions

More information on genetic diversity and relationships within and among cultivated and wild species is required to better understand crop evolution, and to efficiently utilize plant genetic resource collections (Chan and Sun 1997; Xu and Sun 2001). Sauer (1967) suggested two hypotheses concerning the evolutionary origin of grain amaranths. The first hypothesis was that the three grains amaranths were independently domesticated from wild species in three regions; *A. caudatus* from *A. quitensis* in South America, *A. cruentus* from *A. hybridus* in Central America, and *A. hypochondriacus* from *A. powellii* in Mexico. The second hypothesis was that all three amaranth grains share a single origin, and were domesticated from *A. hybridus* in Central America. Previously, the evolutionary relationship among the cultivated and wild *Amaranth* species has been studied using RAPDs and isozymes (Chan and Sun 1997), AFLPs and ISSRs (Xu and Sun 2001), and micromorphology (Costea et al. 2006). However, none of these studies provided conclusive evidence for either hypothesis.

Many researchers have reviewed the potential of the *GBSSI* gene as a useful source of information to resolve phylogenetic relationships in various plant families, including the Poaceae (Mason-Gamer et al. 1998; Ingram and Doyle 2003), Rosaceae (Evans et al. 2000), Araliaceae (Mitchell and Wen 2004), and Adoxaceae (Winkworth and Donoghue 2004). In addition, the origin and evolution of several crops with waxy endosperm has been studied using sequence-based molecular phylogenetic analyses of this gene (Olsen and Schaal, 1999; Domon et al 2002b; Kawase et al. 2004; Fan et al. 2008; Park et al. 2010).

The polymorphisms in sequences of alleles of a single-copy gene can provide useful information about allele genealogies, because the high levels of variation in the non-coding regions provide phylogenetically informative characters. This character was particularly useful in the phylogenetic analysis of *A. hypochondriacus* (Chapter 5). Thus, phylogenetic analysis using sequence information from the *GBSSI* gene may help to define genetic similarities among cultivated species and their wild relatives. Therefore, to explain the interspecific relationships in *Amaranthus*, future studies will

clarify this important issue using the *GBSSI* gene from diverse genetic resources of *Amaranthus*.

6.7 Final comments

Amaranths were first used as a grain crop more than 6,000 years ago in Central America. Grain amaranths have agronomic potential because of the high protein content of the leaves and seeds, and the high levels of the essential amino acid, lysine, in the protein. This crop, therefore, is one of the new world super grains and is gaining favor among health-conscious consumers in many countries, including Japan.

Currently, there is a great deal of interest in basic and applied starch research. Amaranth starch has many unusual properties, including the extremely small granules, low amylose content, a unique dodecahedral structure, stable viscosity, good clarity, and excellent moisture retention. Thus, this starch and its derivatives have potential applications in both food- and non-food industries. Molecular information on the starch properties of this crop plant is required to develop new and useful products. In this respect, the present study is good first step for facilitating further studies on amaranth starch. As well, these results shed new light on our understanding of amylose synthesis.

To date, many scientific and technical articles dealing with the properties, manufacture, composition, economic, and uses of starch derivatives have been published, but only a few of such publications have paid attention to amaranth starch. We still have an incomplete understanding of starch biosynthesis; in particular, the relationship between the structure and function of starches from grain amaranths is still poorly understood. New technologies and agricultural strategies should aim to address this important issue.

6.8 References

Cai X, Wang Z, Xing Y, Zhang J, Hong M (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5'UTR and decreased expression of *waxy* gene in rice cultivars of intermediate amylose content. Plant J 14:459-465

Chan KF, Sun M (1997) Genetic diversity detected by isozyme and RAPD analysis of crop and wild species of amaranthus. Theor Appl Genet 95:865-873

Costea M, Sanders A, Waines G (2001) Preliminary results toward a revision of the *Amaranthus hybridus* species complex (Amaranthaceae). Sida 19:931-974

Costea M, Brenner DM, Tardif FJ, Tan YF, Sun M (2006) Delimitation of *Amaranthus cruentus* L. and *Amaranthus caudatus* L. using micromorphology and AFLP analysis: an application in germplasm identification. Genet Resour Crop Evol 53:1625-1633

Denyer K, Barber LM, Edwards EA, Smith AM, Wang TL (1997) Two isoforms of the GBSSI class of granule-bound starch synthase are differentially expressed in the pea plant (Pisum sativum L.). Plant Cell Environ 20:1566-1572

Dian W, Jiang H, Wu P (2005) Evolution and expression analysis of starch synthase III and IV in rice. J Exp Bot 56:623-632

Domon E, Saito A, Takeda K (2002b) Comparison of the waxy locus sequence from a non-waxy strain and two waxy mutants of spontaneous and artificial origins in barley. Genes Genet Syst 77:351-359

Dry I, Smith A, Edwards A, Bhattacharyya M, Dunn P, Martin C (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. Plant J 2:193-202

Edwards A, Vincken JP, Suurs LC, Visser RG, Zeeman S, Smith A, Martin C (2002) Discrete forms of amylose are synthesized by isoforms of GBSSI in pea. Plant Cell 14:1767-1785

Evans RC, Alice LA, Campbell CS, Kellogg EA, Dickinson TA (2000) The granule-bound starch synthase (GBSSI) gene in the rosaceae: multiple loci and phylogenetic utility. Mol Phylogenet Evol 17:388-400.

Fan LJ, Quan LY, Leng XD, Guo XY, Hu WM, Ruan SL, Ma HS, Zeng MQ (2008) Molecular evidence for post-domestication selection in the Waxy gene of Chinese waxy maize. Mol Breeding 22:329-338

Fedoroff N, Wessler S, Shure M (1983) Isolation of the transposable maize controlling

elements Ac and Ds. Cell 35:235-242

Fukunaga K, Kawase M, Kato K (2002) Structural variation in the *Waxy* gene and differentiation in foxtail millet [*Setaria italica* (L.) P. Beauv.]: implications for multiple origins of the waxy phenotype. Mol Genet Genomics 268:214-222

Furukawa K, Tagaya M, Inouye M, Preiss J, Fukui T (1990) Identification of lysine 15 at the active site in *Escherichia coli* glycogen synthase. Conservation of Lys-X-Gly-Gly sequence in the bacterial and mammalian enzymes. J Biol Chem 265:2086-2090

Furukawa K, Tagaya M, Tanizawa K, Fukui T (1993) Role of the conserved Lys-X-Gly-Gly sequence at the ADP-glucose-binding site in *Escherichia coli* glycogen synthase. J Biol Chem 268:23837-23842

Hirano HY, Eiguchi M, Sano Y (1998) A single base change altered the regulation of the *waxy* gene at the posttranscriptional level during the domestication of rice. Mol Biol Evol 15:978-987

Hirose T, Terao T (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). Planta 220:9-16

Ingram AL, Doyle JJ (2004) Is eragrostis (Poaceae) monophyletic? Insights from nuclear and plastid sequence data. Syst Bot 29:545-552

Isshiki M, Yamamoto Y, Satoh H, Shimamoto K (2001) Nonsense-mediated decay of mutant *waxy* mRNA in rice. Plant Physiol 125:1388-1395

Kawase M, Fukunaga K, Kato K (2005) Diverse origins of waxy foxtail millet crops in East and Southeast Asia mediated by multiple transposable element insertions. Mol Genet Genomics 274:131-140

Kimura T, Ideta O, Saito A (2000) Identification of the gene encoding granule-bound starch synthase I in sweet potato (*Ipomoea batatas* (L.) Lam.). Plant Biotechnol 17:247-252

Klosgen R, Gierl A, Schwartz-Sommer Z, Saedler H (1986) Molecular analysis of the waxy locus of maize. Mol Gen Genet 203:237-244

Konishi Y, Nojima H, Okuno K, Asaoka M, Fuwa H (1985) Characterization of starch granules from waxy, nonwaxy, and hybrid seeds of *Amaranthus hypochondriacus*

L. Agri Biol Chem 49:1965-1971

Lanoue KZ, Wolf PG, Browning S, Hood EE (1996) Phylogenetic analysis of restriction-site variation in wild and cultivated *Amaranthus* species (Amaranthaceae). Theor Appl Genet 93:722-732

Mason-Gamer RJ, Weil C, Kellogg EA (1998) Granule-bound starch synthase: structure, function, and phylogenetic utility. Mol Biol Evol 15:1658-1673

Mitchell A, Wen J (2004) Phylogenetic utility and evidence for multiple copies of granule-bound starch synthase I (GBSSI) in Araliaceae. Taxon 53:29-44

Nakamura T, Vrinten P, Hayakawa K, Ikeda J (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol 118:451-459

Ohdan T, Francisco PB Jr, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y (2005) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. J Exp Bot 56:3229-3244

Okagaki R (1992) Nucleotide sequence of a long cDNA from the rice *waxy* gene. Plant Mol Biol 19:513-516

Okuno K, Sakaguchi S (1981) Glutinous and non-glutinous starches in perisperm of grain amaranths. Cereal Res Commun 9:305-310

Okuno K, Sakaguchi S (1982) Inheritance of starch characteristics in perisperm of Amaranthus hypochondriacus. J Heredity 73:467

Olsen KM, Purugganan MD (2002) Molecular evidence on the origin and evolution of glutinous rice. Genetics 162:941-950

Park YJ, Nemoto K, Nishikawa T, Matsushima K, Minami M, Kawase M (2009) Molecular cloning and characterization of granule bound starch synthase I cDNA from a grain amaranth (*Amaranthus cruentus* L.). Breed Sci 59: 351-360

Sakamoto S (1997) Origin and ethnobotany of glutious perisperm starch found in a species of grain amaranths, *Amaranthus hypochondriacus* L.. Intercultural Studies, Ryukoku University 1:124-133

Sauer JD (1950) The grain amaranths: a survey of their history and classification. Ann Missouri Bot Gard 37:561-619

Sauer JD (1967) The grain amaranths and their relatives: a revised taxonomic and geographic survey. Ann Missouri Bot Gard 54:103-137

Van der Leij F, Visser R, Ponstein A, Jacobsen E, Feenstra W (1991) Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. Mol Gen Genet 228:240-248

Vrinten P, Nakamura T, Yamamori M (1999) Molecular characterization of waxy mutations in wheat. Mol Genet Genomics 261:463-471

Wang Z, Zheng F, Shen G, Gao J, Snustad D, Li M, Zhang J, Hong M (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. Plant Jour 7:613-622

Wessler S, Varagona M (1985) Molecular basis of mutations at the waxy locus of maize: correlation with the fine structure genetic map. Proc Natl Acad Sci 82:4177-4181

Winkworth RC, Donoghue MJ (2004) Viburnum phylogeny: evidence from the duplicated nuclear gene GBSS. Mol Phylogenet Evol 33:109-126

Xu F, Sun M (2001) Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (Amaranthus; Amaranthaceae) using internal transcribedspacer, amplified fragment length polymorphism, and double-primer fluorescent intersimple sequence repeat markers. Mol Phylogenet Evol 21:372-387

Yan H, Jiang H, Pan X, Li M, Chen Y, Wu G (2009) The gene encoding starch synthase IIc exists in maize and wheat. Plant Sci 176:51-57

Acknowledgements

I would like to give heartful thanks to Prof. Dr. Minami who provided carefully considered feedback and valuable comments. I am also indebt to Assoiate Prof. Matsushima and Dr. Park whose meticulous comments were an enormous help to me. I would also like to express my gratitude to my family for their moral support and warm encouragements.

Sammary

Generally, the amylose content of storage starch is considered as one of the most important quality parameters in cereals. As a key player in amylose synthesis, GBSSI plays a major part in determining the functionality of starch in food or non-food industries. In this respect, GBSSI was one of the first plant genes cloned, and is agriculturally important in many crops. As in other cereals, the amylose content of amaranth grain plays important roles in its cooking properties, grain yield, palatability, processing quality, and other agronomic traits. However, amaranth starches have been under-used in several industry sectors because of the lack of research and/or knowledge about their functional properties at the molecular level. Therefore, the aim of this research was to facilitate further studies on amaranth starch through an understanding of the genetic characteristics of the *GBSSI* gene and the molecular mechanism of its mutants.

Three *Amaranth* species show waxy phenotypes that arose from mutation of the *Waxy* to the *waxy* allele. Three genes encoding the waxy protein were isolated from *A. caudatus* (*Wx-ca*), *A. cruentus* (*Wx-cr*), and *A. hypochondriacus* (*Wx-hy*). Sequence analysis indicated that the *Wx-ca*, *Wx-cr*, and *Wx-hy* genes contained the same exon (13 exons) and intron (12 introns) structure. The alignment of the coding sequence of the three *Waxy* genes showed 12 polymorphic sites including 11 SNPs (in exons 10 and 12, in introns 1, 3, 4, 9, and 11) and 5 indels (in introns 4, 9 and 11). In particular, a major polymorphism was detected in intron 4 (8 bp and 3 bp indels). The mutation in the *waxy* alleles (*wx-ca*, *wx-cr*, and *wx-hy*) of all three species was isolated and characterized. Comparison of coding sequences of the three *Waxy* genes and their *waxy* alleles revealed one base insertion (*wx-ca*: insertion of T in exon 8) and a base substitution (*wx-cr*: a G to T base substitution in exon 10, *wx-hy*: a G to A base substitution in exon 6). These nonsense or frame shift mutations introduced an internal termination codon in the three *Waxy* genes. Therefore, these different mutations in the coding regions were considered to be the cause of the waxy (amylose-free) phenotype (Chapter 3).

The existence of polymorphisms in a large gene pool of the *Waxy* locus was

investigated for an origin-and-evolution study. Samples were obtained from 53 strains of *A. hypochondriacus* with various waxy phenotypes collected from different regions. PCR-RFLP or/and direct sequence analysis were used to screen these strains for the G-A polymorphism in exon 6. The results showed that the nonsense mutation in the coding region (exon 6) of the *Waxy* gene was responsible for the change in perisperm starch, leading to a waxy phenotype in all strains. The phylogenetic analysis, which was based on the *Waxy* variation, indicated that diverse waxy types occurred separately and independently in certain domesticated regions in Mexico. In this study, nine molecular types were identified by comparing coding regions of the structural gene of *Waxy* varieties. Among these nine molecular types, *A. hypochondriacus* contained Type III, which could be divided into three subtypes in which the waxy phenotype originated from the Type II (that is, the G-A polymorphism). In addition, these types had the same mutation point that resulted in conversion of the *Waxy* into the *waxy* allele, giving rise to the waxy phenotype. Therefore, the results of the present study showed that this nonsense mutation is a unique event in the evolution of the waxy *Amaranth* phenotypes (Chapter 4).

We also investigated the genetic diversity of *GBSSI* among 37 strains of grain amaranth originating from the New World. A comparison of the *GBSSI* coding sequence revealed an extremely high level of sequence conservation, and a single nucleotide polymorphism between the sequences of non-waxy (Type I) and waxy (Type II) phenotypes. This indicates that a G–T polymorphism in exon 10 (a nonsense mutation) was a unique event in the evolution of the *GBSSI* gene in amaranth grains. This detected SNP was also used to develop an AS-PCR marker for the genetic differentiation between *Waxy* and *waxy* alleles in this crop. The result clearly showed that *GBSSI* allele-specific primers based on this SNP could reliably differentiate the two alleles, *Waxy* and *waxy* (Chapter 5).

This study provides useful information about *GBSSI* expression and GBSSI protein levels, and information about various mutations, evolution, and the contribution of this enzyme to amylose synthesis. In addition, a basic understanding of these characteristics will contribute to facilitating further studies on amaranth starch.