Morphometry analysis and seed germination of *Amaranthus cruentus*, *A. retroflexus* and their hybrid (*A.* × *turicensis*)

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ABSTRACT

A morphometric study of *Amaranthus cruentus*, *A. retroflexus* and their hybrid, *A.* × *turicensis* based on 75 plant samples (750 inflorescences), collected throughout the Olomouc-Holice area (Czech Republic), is presented. Using multivariete methods (including cluster analysis and canonical discriminant analysis), the existence of three groupings of plants was proven. The hybrid exhibited intermediate values of the width and length of female tepals, length of awl-shaped bracts, and seed size when compared with parental species. A germination experiment showed that dark seeds of *A.* × *turicensis* as well as dark seeds of *A. retroflexus* germinate scarcely and independently on the day length while light seeds of *A. cruentus* germinate promptly and markedly better under a short day regime. The chromosome analysis showed that *A. retroflexus*, *A. cruentus*, and *A.* × *turicensis* have the same chromosome number 34.

Keywords: amaranth; chromosome; morphological characteristics; seed germination; spontaneous hybrids

Farmers have been interested in the resurgence of grain amaranth as a new crop for the last few decades (Stallknecht and Schulz-Schaeffer 1993). However, grain amaranth is an ancient crop that was cultivated as early as 5000 to 7000 years ago (Sauer 1967). The evolution of grain species is connected with introgressive hybridisation between cultivated and weedy amaranths (Kulakow and Hauptli 1994). The same mechanisms were involved during the historical evolution of domestic species (*A. hybridus*, *A. hypochondriacus*, *A. caudatus* and *A. cruentus*). The crop originated approximately 5000–6000 years ago as a crop-weed complex in subtropical and tropical areas in America, Asia, and Africa. Both designed and spontaneous inter-specific hybrids of cultivated and weedy amaranths were usually handicapped by high sterility of the first (familiar) hybrid generation (Prizster 1958, Kulakow and Hauptli 1994) which resulted in a low production of dark seeds (Weber et al. 1990). Nevertheless, spontaneous introgression between weedy and facultatively foreign-pollinated domestic amaranths played an important role in the domestication processes. Therefore, the wild species could be considered as a perspective tertiary genetic pool of amaranth (Harlan 1985).

Since 1994, the cultivation experiments with amaranths have been carried out in the Czech Republic in cooperation with the Bohemia Amaranth company, The Research Institute of Crop Production (Prague-Ruzyně), and The Czech Agricultural University. The main purpose of this project was to find regions suitable for amaranth cultivation and to elaborate methods for the cultivation processes (Moudrý 2001). Amaranth species *A. hypochondriacus*, *A. hybridus*, *A. cruentus* are cultivated for grain production in about 1000 ha plots in the whole Europe, out of which approximately one quarter is found in the Czech Republic. However, closely related non-crop amaranths, which are able to hybridise with cultivated amaranth species, also occur as invasive weeds in cultivated fields throughout the country. The naturalisation history of the weedy amaranth species in the country is about two centuries long. During the time, some variability may have evolved (gene pool of a new crop-weed complex) different from the gene pool in the evolutionary centers in tropical and subtropical America, Africa and Asia.

We studied morphometric traits and seed viability in sympatrically occurring populations of *A. cruentus* and *A. retroflexus* and their hybrid *A.* × *turicensis,* all co-occurring in the Olomouc-Holice area where amaranth has been under cultivation since 1994 until now. We compared morphometry traits of the hybrid amaranth with its species, and quantified the seed ability of germination under different light regimes across the two species and their partially fertile hybrid (see Prizster 1958, Jehlík 1990). In addition, we performed chromosome analysis to evaluate any difference in the number of chromosomes between the parents and their hybrid.

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MATERIAL AND METHODS

Nomenclature. For distinguishing both parental amaranth species and their hybrid, the key and nomenclature of Jehlík (1990) was used.

Effect of day-length regimes on seed germination. The experiment was conducted in growth chambers using seeds of *A. retroflexus, A. cruentus* and *A. × turicensis*. Seeds were collected in two locations to ensure the use of clear parental genotypes for the experiment. Seeds of *A. retroflexus* were collected in the locality Jičín, Northeast Bohemia, where no other amaranth species was observed. Seeds of *A. cruentus* (variety AMAR-2RR) were obtained from the Gene Bank Institute (Olomouc), and hybrid seeds of *A. × turicensis* were collected in the Olomouc-Holice area from the plants used for morphometric analysis and other hybrid plants growing there. Prior to the chamber experiment, the seeds were not treated and were stored at 4°C for 8 weeks. After this, 100 seeds of each species were sown in a regular grid on a watered tissue paper in 14 × 7 cm plastic boxes closed by a lid. Eight plastic boxes of each species were placed in a completely randomized design into two separated growth chambers in which the day/night photoperiod of 8/16 (short day, SD) and 12/12 (long day, LD), respectively, were established. Photoperiod was adjusted by two 40 W incandescent bulbs that provided approximately 40 μmol/m^2/s photosynthetic photon flux densities. This level of irradiance emitted from the bulbs was high enough to affect the photoperiod response (Tollenaar 1999). The number of emerging seedlings was counted 5 days after establishing the experiment.

Effect of SAVO treatment on seed-germination. Because seeds of *A. × turicensis* germinated poorly under the standard photoperiod regimes described above, we decided to treat them with a SAVO preparation (5% solution of NaClO) in another chamber experiment. The clearing of the seed coat by SAVO enhances the water intake by endosperm and embryo (Ellis et al. 1985). The seeds were put into SAVO that was shaken to ensure a seed submersion. After 45 min, they were removed and rinsed with distilled water. In the next step, 100 seeds of each species (8 replicates) were put in a regular grid into boxes which were carefully enclosed, and than placed in a completely randomized design in a chamber with the LD photoperiod. Seed germination was recorded after 3 days from the start of the experiment..

Multivariate morphometric study. For the purpose of the morphometric analyses, 25 mature plants of each species (*A. retroflexus*, *A. cruentus* and *A. × turicensis*) were collected in the Olomouc-Holice area; from each of the species we selected 250 inflorescences. The following quantitative traits were measured for each florescence: width of female tepals (WT), length of female tepals (LT), length of awl-shaped bracts (LB), and seed size (SZ). We selected these criteria because they are relatively uniform when compared with such plastic traits as length of leaves, number of leaves, or length of stem (see Silvertown and Doust 1993).

Chromosomes analysis. Chromosomes of *A. retroflexus*, *A. cruentus* and *A. × turicensis* were stained with tolluidine blue (Joachimiak 1994): collected root tips of one-week-old seedlings were pretreated for 12 hours with 2% colchicine, than macerated with 5N HCl at temperature of 50°C, transferred on a slide and squashed in 45% acetic acid, than frozen by liquid nitrogen. The slide with the plant material was covered with a drop of 1% solution of tolluidine blue and stained for 30 seconds, than washed in distilled water and 96% ethanol. Five to seven squash preparations per each species were observed by light microscopy and micrograph (Joachimiak 1994).

Data analysis. Two-way and one-way ANOVA models in STATISTICA (Anonym 1996) were used to analyse the data from the both chamber experiments. Data were arcsine transformed prior to analyses to achieve normality. The morphometrical data were analysed as follows. First, cluster analysis was carried out via the method of unweighted pair-group averaging (UPGMA) in order to check for the possible appurtenance of the individual plants to either the parental species (*A. retroflexus* or *A. cruentus*) or to the expected hybrid (*A. × turicensis*). Second, to evaluate relationships between the traits, two correlation coefficients, Pearson and Spearman, were calculated on the matrix of the total material and on the matrices of species groups of parental taxa and their expected hybrid. Finally, canonical discriminant analysis was performed as a hypothesis-testing method. All numerical analyses were performed using the STATISTICA package.

RESULTS

Germination of *A. retroflexus*, *A. cruentus* and their hybrid *A. × turicensis*

Dark coloured seeds of *A. retroflexus* germinated poorly, their germination was not affected by the photoperi-

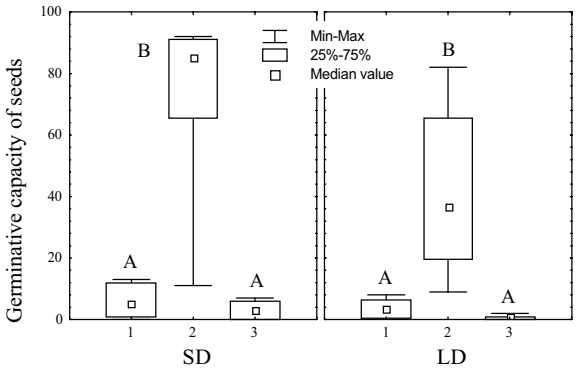

Figure 1. Germination in per cent of three selected species under the day-length condition; box and whisker plots with the same uppercase letter are not significantly different (Tukey *hsd*, $P > 0.05$); 1 = *A. retroflexus*, 2 = *A. cruentus*, 3 = *A. × turicensis*; SD = short day, LD = long day

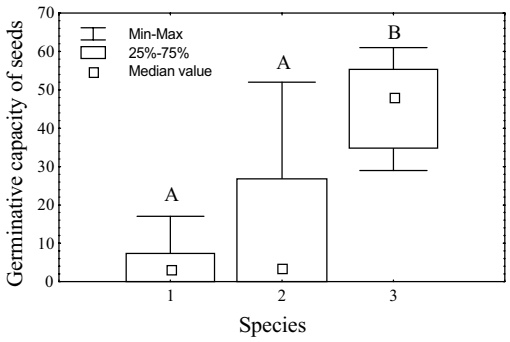

Figure 2. Germination in per cent of three selected species after breaking dormancy by SAVO-bleach; box and whisker plots with the same uppercase letter are not significantly different (Tukey *hsd*, $P > 0.05$); 1 = *A. retroflexus*, 2 = *A. cruentus*, 3 = *A.* × *turicensis*

od (treatments: SD and LD: $F = 1.17$, $df = 1$, $P = 0.297$). Light yellow coloured seeds of *A. cruentus* germinated instantly and expressively on short day (SD vs LD: $F = 5.02$, $df = 1$, $p < 0.05$). Black colored seeds of *A.* × *turicensis* germinated better under short day conditions but the effect was not statistically significant ($F = 2.28$, $df = 1$, $P = 0.15$) (Figure 1). The mean seed germination was 6.1%, 73.3%, 3.1% at SD, and 3.6%, 41.8%, 0.8% at LD for *A. retroflexus*, *A. cruentus* and *A.* × *turicensis*, respectively. Dormancy of black seeds was broken up by SAVO bleaching (see Figure 2) which significantly enhanced the germination of all the three selected amaranth species ($F = 20.41$, $df = 2$, $p < 0.001$).

The morphometrics

1. On the UPGMA dendrogram, three main clusters can be distinguished (Figure 3). The first of them contains individuals that can be classified as pure *A. retroflexus*; the second one contains individuals classifiable as pure *A. cruentus*. In the third cluster, intermediate individuals are clustered together, most probably representing hybrids. It is apparent from the background data (Table 1) that the hybrid individuals exhibited intermediate values of morphometric traits in all cases.
2. As revealed by the correlation coefficients calculated for the total material, no pair of traits was very highly correlated (exceeding value 0.95) or highly correlated (exceeding value 0.9). The highest correlation coefficient was 0.76. It follows that all the traits could be used in the discriminant analysis.
3. Three groupings were also apparent on the ordination diagram (Figure 4) resulting from the canonical discriminant analysis using individual inflorescences as operational taxonomic units (OTUs). The groupings were arranged along the first canonical axis which accounted for almost 86.8% of the total variation. In spite of the fact that the groupings were separated by visible gaps, there were only small overlaps among them. Based on the results of the classificatory analysis (Table 2), the correct classification of the hybrid *A.* × *turicensis* was less frequent.

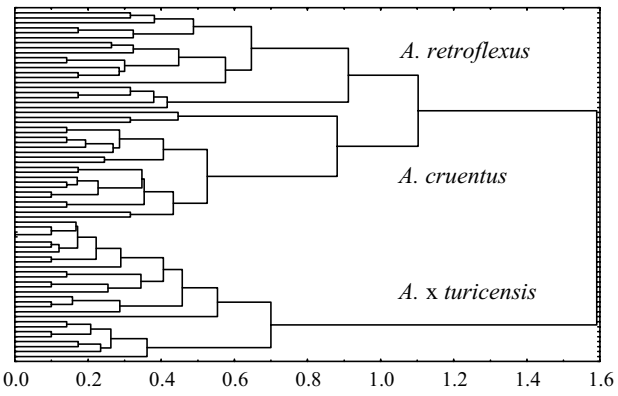

Figure 3. Cluster analysis of *Amaranthus retroflexus*, *A. cruentus* and *A.* × *turicenis* – UPGMA method

Table 1. Mean values, percentiles (5%, 95%) and standard error for quantitative characters of *Amaranthus retroflexus*, *A. cruentus* and *A.* × *turicensis*

Characters (mm)	Group	Mean	5%	95%	Standard error
LB	RET	4.24	4.15	4.34	0.05
	CRU	2.55	2.49	2.59	0.03
	TUR	3.82	3.74	3.89	0.04
LT	RET	2.84	2.77	2.90	0.03
	CRU	1.71	1.68	1.74	0.02
	TUR	1.82	1.78	1.85	0.02
W T	RET	0.74	0.72	0.75	0.01
	CRU	0.54	0.53	0.56	0.01
	TUR	0.63	0.62	0.65	0.01
SZ	RET	1.11	1.09	1.12	0.01
	CRU	1.32	1.30	1.33	0.01
	TUR	1.27	1.26	1.28	0.01

LB = length of awl-shaped bracts, LT = length of female tepals, WT = width of female tepals, SZ = seed-size
RET = *Amaranthus retroflexus*, CRU = *A. cruentus*, TUR = *A.* × *turicensis*

Chromosome counts

The species *A. retroflexus*, *A. cruentus* and *A.* × *turicensis* had the same chromosome number 34. No higher level of ploidity was detected. Chromosomes of all species studied are uniform, short, and monotypic. No

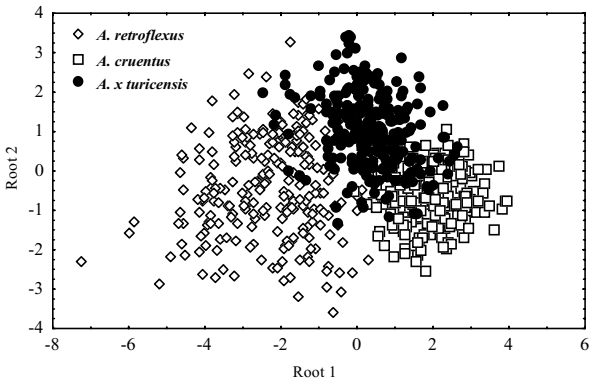

Figure 4. Discriminant analysis of selected morphological traits separating three amaranth species ($F_{8.1488}$ = 275.11, $P << 0.001$)

marked differences in chromosome counts and visual aspects (length, centromere position) were observed.

DISCUSSION

Manipulative germination experiments reveal that seeds of the weedy species *A. retroflexus* and hybrid *A.* × *turicensis* are dormant while seeds of the cultivated species *A. cruentus* are affected by the photoperiod and germinate expressively under short-day regime. This suggests that light yellow seeds of cultivated species *A. cruentus* are probably more sensitive to the photoperiod. On the contrary, Thomas and Vince-Prue (1984) and Huang et al. (2000) found that amaranth seedlings are insensitive to the photoperiod during the juvenile phase of the plant growth. The possible explanation for this is the fact that cultivated amaranths were selected for quick seed-germination rate mainly under the short-day condition (Espitia-Rangel 1994). The seed colour *A.* × *turicensis* varies from brown to black (character inherited after *A. retroflexus*). Dark colour is typical for a hard seed coat, which is an important trait for the creation of a persistent seed bank (Barton 1961). Thus, hybrid seeds are crucial for the delayed field germination. Our study showed that it is possible to enhance germination of the hybrid seeds by using the SAVO-bleach treatment. Seeds of weedy *A. retroflexus* were not very strongly affected by the treatment whereas the seed coat of *A. cruentus* was quickly eroded and the SAVO-bleach probably penetrated into the plant embryo thus resulting in very poor seed germination.

Table 2. Results of classificatory discriminant analysis of *Amaranthus retroflexus* (RET), *A. cruentus* (CRU) and *A.* × *turicensis* (TUR)

	Predicted group membership		
Actual group	RET	CRU	TUR
RET	**222**~88.8	**3**~1.2	**25**~10.0
CRU	**0**~00	**237**~94.8	**13**~5.2
TUR	**9**~3.6	**34**~13.6	**207**~82.8

number of observations~percentage classified into groups

As it was shown by morphometric analysis, three groups of individuals occurred at the Olomouc-Holice locality. All hybrid morphological characteristics measured were intermediate when compared with parental species. However, hybrid plants usually exhibit a remarkable heterosis phenomenon in some characters such as the length and the width of leaves and the length of stems (Weber et al. 1990). In the field, we found some hybrid plants with partly or completely red-coloured vegetative organs. It is highly possible that the type of colouration of vegetative organs can be affected by the cultivated variety which hybridises with weed species. It was also of interest that the hybrid plants had relatively rich branching stems. This is most likely inherited from *A. retroflexus*, a species with a similar branching pattern in low competiton habitats (Frey 1974, Weaver and McWilliams 1980, Jehlík 1990, but see Speranza and Bignami 1997). In contrast, *A. cruentus* has been bred for the inflorescence reduction and for a simple erect stem (Espitia-Rangel 1994). In many fields of cultivated amaranths, naturally occurring crop-weed hybrids are common (Kulakow and Hauptli 1994). The first occurrence of hybrids *A.* × *turicensis* in Europe since 1907 is mentioned already in a Priszter's study (1958), who intensively studied this taxonomically difficult group of plants. Later hybrid occurrence in human-modified habitats is connected with increasing commercial cultivation of amaranths (Weber et al. 1990).

Recent species of the genus *Amaranthus* are polyploids (basic number $x = 8$) and the chromosome number $n = 17$ originated later by primary trisomy (Greizerstein and Poggio 1992). Our evaluation of the chromosome analysis of *A.* × *turicensis* hybrid ($2n = 34$) showed that both parental species (*A. cruentus* and *A. retroflexus*) should hybridise relatively easily. However, most of the amaranth hybrids exhibit relatively high levels of sterility which was already confirmed by Gupta and Gudu (1991). It is generally known that a single cultivated plant is able to produce as many as 50 000–200 000 of seeds per florescence (Jarošová et al. 1998, Popenoe et al. 1989). In contrast, our field observations reveal that the hybrid plants reached on average only 52 seeds at the 32 cm long inflorescence.

Anthropogenic hybridisation and introgression are dramatically becoming worldwide because of the translocations of organisms and habitat modifications by humans. The introduction of cultivated grain amaranths to the conditions of central Europe, where they get into contact with the wild amaranths, is an example of such anthropogenic hybridisation (Allendorf et al. 2001). The main result of the at least 5-year existence of the crop-weed complex of genus *Amaranthus* in Olomouc area is the pollution of amaranth crop yields by the dark hybrid seeds. The introduction of new amaranth crops to the Czech Republic can,

with a high possibility, lead to the evolution of hybrid swarms on a large scale. However, the possibility of colonisation of neighbouring area by hybrid plants calls for further research. At present, it is important to use insulators to control pollination by the amaranth variety multiplication (Brenner and Widerlechner 1998) or to suppress the weedy species (mainly *A. retroflexus*) in the early stage of growth under field conditions (Oryokot et al. 1997).

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ABSTRAKT

Morfometrická analýza a klíčení druhů *Amaranthus cruentus*, *A. retroflexus* a jejich hybridu (*A.* × *turicensis*)

Práce se zabývá morfometrií vybraných znaků druhů *Amaranthus cruentus*, *A. retroflexus* a jejich hybridu *A.* × *turicensis* sbíraných na lokalitě Olomouc-Holice (Česká republika). Ke klasifikaci sebraných rostlin bylo užito shlukovací a diskriminační analýzy, jimiž byl studovaný materiál separován do tří skupin odpovídajících hybridním rostlinám a rostlinám mateřských druhů. Hodnoty vybraných znaků měřené v rámci květu (šířka a délka samičího okvětního lístku, délka listence

a velikost semene) se u hybridu ve srovnání s mateřskými druhy projevovaly intermediánností. Dále bylo zjištěno, že tmavě zbarvená semena hybridu *A.* × *turicensis* a plevelného druhu *A. retroflexus* klíčila nepatrně a nezávisle na délce dne, zatímco světlá semena druhu *A. cruentus* klíčila okamžitě a výrazněji lépe v podmínkách krátkého dne. Počet chromozomů u hybridu i obou mateřských druhů byl stejný a roven 34.

Klíčová slova: amarant; chromozomy; morfologické znaky; klíčivost semen; spontánní hybridy

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