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RESEARCH NOTES

Production and Identification of Interspecific Hybrids between Pepper (*Capsicum annuum* L.) and the Wild Relative (*Capsicum frutescens* L.)

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ABSTRACT

Wild pepper (*Capsicum frutescens* L.) could be a source of variation to improve cultivated pepper due to its unique traits with adapting challenges caused by adversity. Interspecific hybridization has been used as an effective way of pepper introgression breeding, which transfers genes of interest from wild relatives to cultivated crops. Here, eight fertile hybrids F_1 were produced from pepper (*Capsicum annuum* L.) and the wild relative (*C. frutescens*), as female and male, respectively, by interspecific hybridization. Interspecific hybrids were identified using conventional morphological descriptors and SSR molecular markers. The results showed that significant differences in agronomic traits existed among cultivated pepper, wild relatives, and interspecific hybrid F_1. Interspecific hybrid F_1 presented intermediate values, although they were closer to the wild species in most of the agronomic traits. Analysis of SSR markers clearly showed that interspecific hybrid F_1 had bands from the paternal and maternal accessions, which indicated that F_1 hybrid was heterozygous. Our results provide hybrid for breeder to transfer genes of interest from wild relative, *C. frutescens*, to cultivated pepper, which is an important step for introgression breeding.

Keywords: Gene transfer, Interspecific hybridization, Introgression breeding, Morphological descriptors.

INTRODUCTION

Capsicum is native to Central and South America and 5 species of which are domesticated, including *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, *C. pubescens* (Ibiza *et al*., 2012). Of the five domesticated species, *C. annuum* (2n= 2x= 24), a native species in Mexico, is one of the economically important vegetable crops (Wang and Bosland, 2006), which is also the most common and extensively cultivated in the tropical and subtropical regions across the world (Wahyuni *et al*., 2013).

However, the genetic base of cultivated pepper (*C. annuum*) is increasingly narrow under the domestication, and further hinders breeding progress. This narrow genetic base contrasts with large genetic variation present in the pepper wild relatives. It has been reported that the genus *Capsicum* contains over 20 wild species, some of which have been used in breeding for decades. These wild relatives are useful for broadening

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narrow genetic base of cultivated pepper (Honnay *et al*., 2012).

A member of *C. frutescens* (named as *xiaomila*) is naturally distributed in the southern Yunnan Province (Deng *et al*., 2009) and is the only wild germplasm resource in China (Liu *et al*., 2013). Some desirable traits for pepper variety improvement in *xiaomila* also were reported, including resistance to biotic and abiotic stresses. In consequence, these desirable traits from *xiaomila* could be transferred into cultivated pepper to further create new germplasm resources for breeding exploitation.

Interspecific hybridization is widely used for creating new germplasm and expanding genetic diversity in pepper (Yoon *et al*., 2004, 2006). However, successful interspecific crosses were limited in most cases, due to cross incompatibility. Thus, in this respect, most of the studies tent to solve the incompatibility among *Capsicum*. For instance, Yoon *et al*. (2009) reported that anthracnose resistance was successfully introgressed from *C. baccatum* to *C. annuum* using embryo rescue. Furthermore, F_1 hybrids were obtained using an embryo rescue technique in the cross combination *C. annuum* 7033×*C. chinense* 7020 (Sui and Hui, 2015). It is reported that interspecific hybrid has been obtained in cross combination between *C. chinense*×*C. frutescens*, and the pollen viability was devoted to study in their generations (Monteiro *et al*., 2011). Costa *et al*. (2009) reported that the fruit and viable seeds were obtained in interspecific crosses of *C. chinense* with *C. annuum.* Nine seeds were obtained from the combination of C.annuum × C. baccatum, but seven seedlings unsuccessfully reach maturity from the nine hybrids. (Martins *et al*., 2015).

The objective of the present work was to obtain hybrid F_1 from *C. frutescens* (*xiaomila*), a wild relative from Yunnan Province in China, and cultivated *C. annuum* (an excellent breeding line, 007EA). Identification of interspecific hybrid F_1 of *C. annuum* and *C. frutescens* was performed by using phytological traits and SSR markers. The results will provide a bridge for the breeder to carry successful introgressions from a wild *C. frutescens* (*xiaomila*) to cultivated pepper.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Two accessions, the wild *C. frutescens* (*xiaomila*-P1512) from Yunnan Province in China and cultivated pepper (*C. annuum*-007EA), were used in this experiment. The fruits of P1512 are extremely small and pungent and the cultivated pepper 007EA is a landrace from China with large red fruits. This experiment was carried out in the glass greenhouse of Vegetable Research Institute in Zhejiang Academy of Agricultural Science from the autumn of 2015 to the summer of 2016. The greenhouse temperatures were maintained at 20°C minimum and 30°C maximum. (GPS coordinates of the plot: 30° 21′ 5.27″ N, 120° 22′ 56.01″ E).

Production of Interspecific Hybrid F_1

The mature balloon shaped buds of the female parents were emasculated in previous day at anthesis to avoid self-pollination and covered with butter-paper bags. Pollination was made in the morning using freshly mature pollen from flowers collected from male parent. The pollinated flowers were tagged with genotypes of the parents involved in the cross and the date at which it was finished. The rest of buds and the naturally pollinated ones were cut off. Reciprocal crosses to wild *C. frutescens* (*xiaomila*-P1512) were carried out. Overall, more than 1,000 hybridizations were performed in this experiment.

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Identification of Interspecific Hybrid F_1

Morphological Characterization

The seeds of $P_1 \times P_2$ were germinated in combination (*xiaomila*-P1512 as male parental line, 007EA as female parental line), while on fruit set with reciprocal cross while no hybrid was obtained in the opposite direction in 2006. Agronomic traits of cultivated pepper, wild species, and interspecific hybrid F_1 were evaluated with five replications in 2015 and 2016, which described traits of the whole plants, leaf, flower, and fruits. Five measurements were recorded for each agronomic trait to obtain individual plant averages, except for the whole plant height, length of a knot and the main stem, for which one measurement was recorded. One plant per replication was taken for recording the different morphological traits. A total of 22 morphometric descriptors were recorded in this experiment (Supplemental Table 1). Of which ten descriptors (e.g., panel length, leaf length, width and petiole length, pedicel length, corolla diameter, style length, stamens length, fruit width and length) were measured using vernier caliper. Plant height and fruit weight were measured using flexible ruler and electronic scales, respectively.

SSR-PCR Analysis

Approximately 0.2 g leaf samples of each of the wild *C. frutescens* P1512, cultivated pepper 007EA, and interspecific hybrid F_1 were taken out of fridge (-80°C) to a box filled with liquid nitrogen. Then, these samples were ground into powder using a pestle and mortar.

DNA Extraction: The powder was poured into 2-mL centrifuge tube with 1 mL overheated DNA buffer and 2 µL β-mercaptoethanol, which was watered bath at 65°C for half an hour. Next, 750 µL mixture from the 2 mL centrifuge tube was transferred to another new tube, and 750 µL chloroform was poured into the new tube to obtain 1.5 mL mixed liquor, which was oscillated 10-15 minutes and centrifuged for 5 minutes at 12,000 rnp. After that, the supernatant from the new tube was separated into another new tube; the last step was repeated in order to acquire DNA with high purity. Then, the supernatant was taken out and added to isopropyl alcohol (V= 0.7v supernatant), and centrifuged for 10 minutes at 12,000 rnp. Finally, the mixed liquor was poured out and centrifuged at the same speed after adding alcohol twice (Absolute ethyl alcohol: Water= 3:1). Alcohol from the mixed liquor was poured out and placed for air dry. Finally, the DNA was dissolved in 50 µL ddH_2O and kept in -20°C refrigerator as reserve. DNA purity was tested with 1% agarose gel electrophoresis.

PCR Amplification: The reaction mixture was conducted using PCR thermocycler (BioRad, MJ Mini). A total of 20 µL PCR reaction volumes contains 1 µL DNA template, 10 µL 2x Taq Plus Master Mix, 2 µL positive and negative SSR primers. In addition, 7 µL ddH_2O was added to the total volume. PCR amplification was began by initial denaturation at 94°C for 5 minutes, then, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The amplification was accomplished by maintaining the reaction mixture at 72°C for 7 minutes, to allow full extension of PCR products.

SSR Marker: PCR products were separated by using a 10% PAGE gel electrophoresis under 200V for 2 hours. PAGE gel was stained with 0.5 g L^{-1} silver nitrate for 5 to 10 minutes, and shaken10 minutes in the developer (consisting of 15 g L^{-1} NaOH, 0.25 g L^{-1} $Na_2B_4O_7 \cdot 10H_2O$ and 4 mL L^{-1} CH_2O). After that, PAGE gel was washed with distilled water till a clear band was observed. Finally, PAGE gel was wrapped up with a fresh keeping film. Meanwhile, PCR products with ethidium bromide were run by 3% agarose gel electrophoresis, under 110V/50 Ma set up for half hour, and were

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photographed and recorded using a gel imaging system.

RESULTS

Production of F_1 Hybrid

Twenty-five flowers per plant were pollinated with each of the parents to ensure the fruit set. Furthermore, to increase fruit setting rate in budding period, pollination was repeated and each stigma was coated with pollen derived from three male flowers. Totally, 431 seeds were obtained in this experiment. These seeds were cleaned and dried, and sowed in plastic basins. Only eight F_1 hybrids survived in the end, which were transplanted in greenhouse at the third true leaf stage, and properly managed until the fruits ripping.

Morphological Traits in Parental Lines and F_1 Hybrids

Obvious differences in plant morphological characters were observed among cultivated pepper (007EA), wild relative (*C. frutescens* P1512), and interspecific hybrid F_1. For most morphological characteristics, interspecific hybrid was closer to the wild species compared to the cultivated species. These traits included plant height, the internode length, leaf length, leaf width, leaf petiole length, petal number, corolla diameter, stamens length, fruit weight, fruit length, and fruit diameter (Supplemental Table 2; Figure 1). These traits had smaller values in wild species and interspecific hybrid, with the exception of two traits (plant height, the internode length) that had higher average values (Supplemental Table 2; Figure 1). Furthermore, we also found that interspecific hybrids were extremely similar to the wild species in the following four traits including flower color, pedicel position, leaf and fruit shape. On the contrary, interspecific hybrids were found closer to cultivated species in the fruit position. Moreover, purple anthocyanin accumulated at the nodal positions in the cultivated species and interspecific hybrids, except for the wild species (Supplemental Table 1). In addition, while the interspecific hybrids had an intermediate value in flower size, the traits related to the pedicel and style length had much higher values in the interspecific hybrids compared to the parental lines (Supplemental Table 2; Figure 2). Regarding seeds, there were many seeds with the embryo abortion in

Figure 1. Whole plant height and attitude of cultivated pepper, wild relative and interspecific hybrid F_1. (a) Cultivated pepper (*Capsium annuum*) 007EA, (b) Interspecific hybrid F_1 (*C. annuum*×*C. frutescens*) and (c) Wild relative (*C. frutescens*) P1512.

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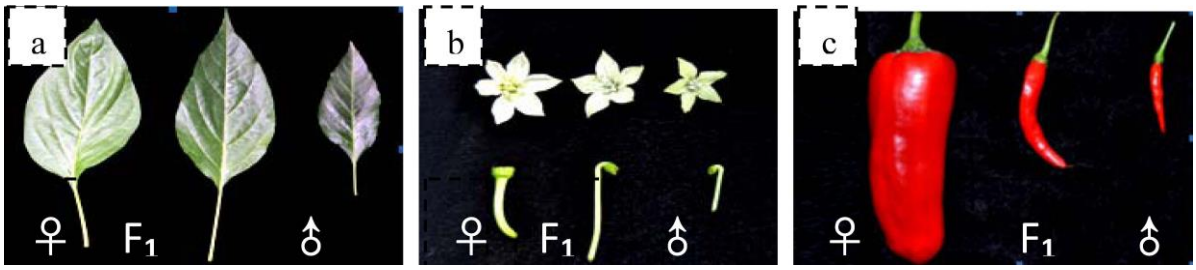

Figure 2. Comparison of leaf, flower and fruit among cultivated pepper (*Capsium annuum*-007EA), wild relative (*C. frutescens*-P1512) and interspecific hybrid F_1. (a) Leaf, (b) Flower and (c) Fruit. (♀: *C. annuum* ,♂: *C. frutescens*).

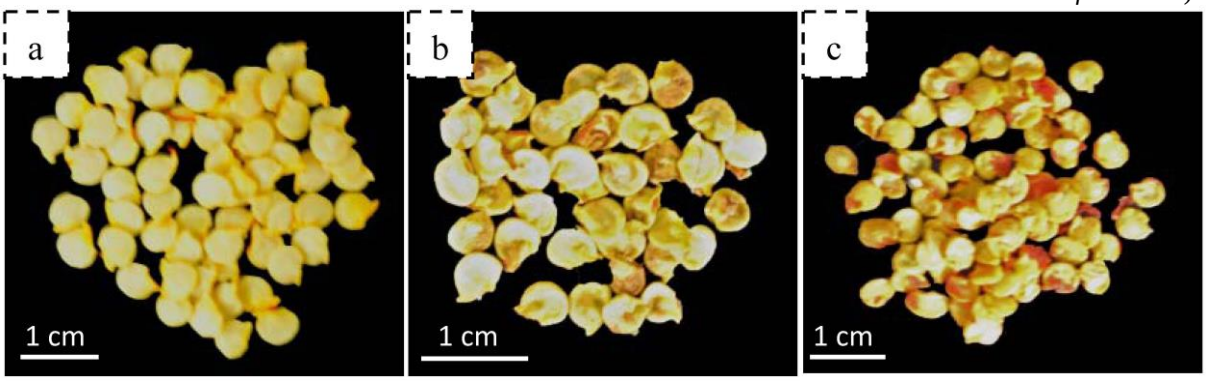

Figure 3. Comparison of seeds among cultivated pepper, wild relative and interspecific hybrid F_1. (a) Cultivated pepper (*Capsium annuum*) 007EA, (b) Interspecific hybrid F_1 (*C. annuum*×*C. frutescens*) and (c) Wild relative (*C. frutescens*) P1512.

the interspecific hybrids, but parental lines showed normal appearances (Supplemental Table 2; Figure 3).

SSR Identification of Interspecific Hybrid F_1

SSR molecular marker was adopted to identify authenticity of hybrid due to high co-dominance, reproducibility, and stability. Thirty-nine pairs of SSR primers were selected randomly in this study. Only one pair of primers (Pe26) could be distinguished among parents and interspecific hybrid F_1 (Supplemental Table 3). The results showed obvious differences of the band patterns among them by the high-resolution of 10% polyacrylamide gel electrophoresis. The differences of the banding patterns between the cultivated pepper and the wild relative (*C. frutescens*) appeared co-dominant in the hybrid F_1 (Figure 4), which indicated the F_1 hybrid was heterozygous. The results were consistent with the morphological identification of interspecific hybrid F_1.

DISCUSSION

Crops wild relatives are recognized as a

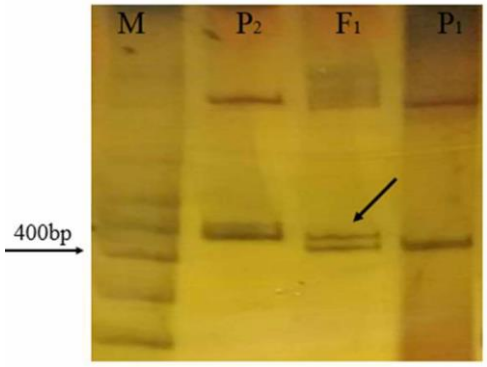

Figure 4. SSR analysis of the banding patterns among the cultivated pepper (P_2), wild relative *C. frutescens* (P_1) and interspecific hybrid F_1. M: Marker, 1,000 bp. The polymorphic bands was indicated by arrows.

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source of variation for good traits in breeding crops. These traits include high tolerance to abiotic and biotic stresses (Hajjar and Hodgkin, 2007; Honnay *et al*., 2012), which can improve crops yield and quality dramatically, adapting them to the serious challenges caused by climate change (Dempewolf *et al*., 2014). One of the most successful examples is tomato, where modern commercial varieties carry many excellent genes from different wild relatives (Lin *et al*., 2014). Pepper (*C. annuum*), one of the most economically important vegetables, is cultivated widely around the world. *C. annuum* is related to about other 30 *Capusicum* species, of which over twenty are wild relatives. These wild species showed abundant variation in plant architecture, leaf-, flower-, and fruit-related traits (Eshbaugh, 1980; Sudré *et al*., 2010; Thul *et al*., 2009). Therefore, broadening the genetic base of cultivated pepper to include excellent genes from wild relatives is vital to developing new varieties.

Interspecific hybridization is one of the most effective ways for breeders to broaden the genetic diversity of cultivated species with narrow genetic base. The success rate of interspecific hybridization is dependent on genetic relationship among different species. Previous researchers have reported that no viable seeds were obtained in crossing C. *annuum* and *C. frutescens*, and a very low percentage of viable seeds in the reciprocal cross (Smith and Heiser, 1951). Cheng *et al*. (2007) reported that although viable seeds were obtained, no progeny survived in the cross *C. annuum*×*C. frutescens*. In this work, interspecific hybrids F_1 were obtained in cross combination of *C. annuum*×*C. frutescens*. These results indicated that both *C. annuum* and *C. frutescens* could present low compatibility and extant genetic relationship. Previous researchers conducted studies to compare the interspecific crossability among 13 different genotypes of 5 cultivated species. The results showed that the cross combination of *C. annuum* with *C. chinense* were compatible, and the crosses of *C. annuum* with *C. frutescens* were partly compatible, and the cross combination of *C. annuum* with *C. baccatum* or *C. pubescens* were completely incompatible (Yoon *et al*., 2004). Our results supported their abovementioned views that the crosses of *C. annuum* with *C. frutescens* were partly compatible.

Actually, only eight interspecific hybrids from more than 431 seeds from the cross between *C. annuum* and *C. frutescens* were obtained in this study. The remaining seeds did not germinate due to embryo abortion (Figure 3). It has been known that embryo rescue is one of the first and successful forms of in vitro culture techniques (Cisneros and Tel-Zur, 2010). Therefore, to obtain more viable interspecific hybrids F_1 between *C. annuum* and *C. frutescens*, embryo rescue technology could be applied before embryo abortion of hybrids. Additionally, it had been reported that *C. chinense* could be utilized as genetic bridge to improve crosses rates between *C. annuum* and *C. frutescens*, *C. baccatum* and *C. pubescens*, respectively (Haque *et al*., 2016; Huang *et al*, 2015; Manzur *et al*., 2015; Rodríguez-Burruezo *et al*, 2010). Thus, *C. chinense* as genetic bridge is the alternative to achieve viable hybrids between *C. annuum* and *C. frutescens*.

In summary, *xiaomila* (*C. frutescens* L) is an excellent wild resource in China (Liu *et al*., 2013), which has many good characteristics, such as tolerance to high temperature and humidity, low light, and poor conditions (Liu *et al*., 2013). In this work, interspecific hybrids F_1 were obtained between *C. annuum* and *C. frutescens*, which could be used as bridge species to transfer genes of interest from *C. frutescens*. Also, combined with backcross introgression method, the good genes from *C. frutescens* will be used to enhance the common pepper cultivar in the future.

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تولید و شناسایی هیبریدهای بین گونه ای فلفل (*Capsicum annuum* L.) و یک خویشاوند وحشی (*Capsicum frutescens* L.)

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چکیده

فلفل وحشی (*Capsicum frutescens* L.) دارای صفات منحصر به فرد سازگاری با چالش های ناشی از شرایط نامناسب است و می تواند منبعی از تنوع و تغییر برای بهبود فلفل معمولی باشد. به این خاطر، ازدورگ گیری بین گونه ای به عنوان روشی موثر در اصلاح نژاد فلفل با رگرسیون حائلی (introgression breeding) که ژن های مطلوب را از خویشاوندان وحشی به گیاه کشت شده منتقل می کند استفاده شده است. در این آزمایش، با استفاده ازدورگ گیری بین گونه ای، ۸ هیبرید بارو F1از تلاقی فلفل (*C. annuum*) و خویشاوند وحشی آن(*C. frutescens* L.) به ترتیب به عنوان ماده و نر، تولید شد. هیبرید های بین گونه ای با استفاده از توصیف کننده های مورفولوژیک رایج

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و شاخص های مولکولی SSRشناسایی شد. بر پایه نتایج، بین فلفل کشت شده، خویشاوندان وحشی، و هیبرید بین گونه ای F1 تفاوت معناداری در صفات اگرونومیکی وجود داشت. هیبرید بین گونه ایF1 ویژگی های بینابینی داشت هرچند که بیشتر صفات اگرونومیکی هیبرید مقدارشان به مقادیر گونه وحشی نزدیک تر بود. تجزیه تحلیل نشانگرهایSSR به روشنی نشان داد که هیبرید بین گونه ای F1 دارای باندهایی از هر دو نمونه مادری و پدری بود و نشانه ای بود از ناخالص بودن هیبرید F1 (heterozygous). نتایج آزمایش ما برای بهنژادگرانی که می خواهند ژن های مطلوب را از گونه وحشی(*C. frutescens*) به گونه کشت شده منتقل کنند هیبرید فراهم می کند و این گام مهمی است برای روش اصلاح نژاد با رگرسیون حائلی.

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