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# ASSESSMENT OF GENETIC DIVERSITY OF CHILI ROOTSTOCK USING ISSR MARKER

Vo Thi Bich Thuy[1], Huynh Ky[1], Tran Thi Ba[1], Nguyen Loc Hien[1] and Swee Keong Yeap[2]

[1]*College of Agriculture and Applied Biology, Can Tho University, Vietnam*
[2]*Institute of Bioscience, Putra University, Malaysia*

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**ABSTRACT**

*The polymorphism level of chili was studied by using ISSR marker. Polymorphism of sixteen chili varieties was evaluated with 15 ISSR primers. These 15 ISSR primers have generated 136 DNA bands, of these 102 bands are polymorphic, with an average of 9.06 bands per primer. The evaluation of the dendrogram obtained by UPGMA demonstrated the differentiation of all the varieties. Four varieties were separated in distinct group in which showed complete correspondence to its observed characters. DNA profile of 16 chili varieties based on ISSR marker revealed the potential of digital fingerprints of all varieties examined.*

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## 1 INTRODUCTION

Chili pepper (Capsicum), a member of Solanaceae is one of the world largest families of plant kingdom and consists of more than 3,000 species. It is a major spice crop, which has been highly cultivated over the years in different parts of the world for human consumption including cooking, decoration and nutrition supplement for health (Pickersgill, 1998). In this genus, Capsicum includes C. annuum, C. chinense, C. baccatum, C. frutescens, and C. pubescens are consumed worldwide and valued because of their unique color, pungency, and aroma. The names of these species for particular varieties reflect their culinary uses and ripeness. The different forms are used as important ingredients of culinary throughout the world. Besides being used as culinary ingredient, chilies are also widely used in medicinal application. According to Mayan and Aztec civilizations of America, extract from chili fruit was used to treat asthma, cough and sore throat (Bosland, 1996). In Asia, folk medicine used chilies to treat many diseases (fevers, colds, nasal catarrh, high blood pressure, stomach, digestive problem, laryngitis, sore throat, rheumatic and neuralgic pain, skin problems, and shingles). Recently, many researchers showed the potential of chili extract for cancer and mutation treatment (De Mejia *et al.*, 1998; Maoka *et al*., 2001; Morre and Morre, 2006).

To date, many chili varieties plethora of phenotypes and different levels of pungency, color, flavor and aroma have been introduced and well adapted in different parts of Vietnam. For example, the fruit of C. annuum is generally mild and used in curried dishes and salads while those of C. frutescens and C. chinense are highly pungent and used as hot spices. On the other hand, C. annuum cv. 'Ot Kieng' is cultivated as an ornamental although its fruits are edible. However, for the last several years, genetic diversity of Vietnamese chili has been controversially enshrouded due to its taxonomy. The origin and phylogenetic affinities of Vietnamese chilies are still not yet studied and they could not be classified based on its morphology. Thus, this study was carried out using DNA mark-

ers to understand the relationship of inter- and intra- Capsicum species in Vietnam.

DNA markers have been intensively used to study the plant diversity. Previously, protein marker was used to classify eight taxa of chili pepper from India (Panda et al. 1986). Lately, the more simple and cost-effective PCR-based DNA fingerprinting is using random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and ISSR have been utilized to assess the plant biodiversity (Bardakci, 2001). In Capsicum, RAPD has successfully characterized the domestic chili germplasms (Paran et al., 1998; Rodriguez et al., 1999; Walsh and Hoot, 2001; Toquica et al., 2003; Sitthiwong et al., 2005; Adetula, 2006; Bosland and Baral, 2007). Here, the study was reported the use of ISSR fingerprinting technique for estimating the genetic distances of Capsicum cultivated in Vietnam to identify the presence of molecular markers associated with the rootstocks of chili used for cultivation.

## 2 MATERIALS AND METHODS

### 2.1 Plant materials

Ripe fruits of the 16 varieties (Table 1) were collected and seeds extracted from the fruits were sown in pots containing soil and kept in a 50% shaded net house. These collected leaves are for DNA extraction.

**Table 1: List of 16 chili varieties using in this study**

| No | Name | Origin |
|---|---|---|
| 1 | Hiem lai 207 | Viet Nong Company |
| 2 | Sung vang Chau phi | Trung Nong Company |
| 3 | Ot Hiem trang | Cantho City |
| 4 | Ot Hiem xanh | Cantho City |
| 5 | Ot TN588 | Trang Nong Company |
| 6 | Ot TN589 | Trang Nong Company |
| 7 | Ot TN591 | Trang Nong Company |
| 8 | Ot TN592 | Trang Nong Company |
| 9 | Ot TN596 | Trang Nong Company |
| 10 | Ot TN557 | Trang Nong Company |
| 11 | Ot TN598 | Trang Nong Company |
| 12 | Ot TN607 | Trang Nong Company |
| 13 | Ot TN608 | Trang Nong Company |
| 14 | Hiem 01 | Cantho City |
| 15 | Ot Da Lat | Da Lat City |
| 16 | Ot Hiem 27 | Mien Nam Company |

### 2.2 DNA extraction

DNA was extracted from the leaf samples by a modified CTAB method (Taylor and Powell, 1982). In brief, frozen leaves (100 g) were ground in liquid nitrogen-chilled mortar and pestle to fine powder. The powder was then transferred into an eppendorf tube containing 1 mL of pre-warmed (65°C) lysis buffer containing 2% CTAB (Merck), 1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), 50 mM EDTA (pH 8.0) and 2% β-mercaptoethanol. The samples were then incubated at 65°C for 1 h with occasional shaking. After incubation, the samples were let cooling at room temperature, and then 1 mL of chloroform was added to the sample and mixed well. Next, it was followed by centrifugation and re-extraction of the supernatant with 1 mL of chloroform–isoamyl alcohol (24:1, v/v). The solution was centrifuged again and 1 mL of cold isopropyl alcohol was added to the supernatant followed by incubation at −20°C for 15 min followed by centrifugation. The pellet was dried, washed with 70% ethanol and then dissolved in 300 µL of TE buffer and 5 µL RNase (10 mg/mL) and incubated at 37°C for 2 h. The DNA solution was re-extracted two times with equal volume of chloroform–isoamyl alcohol (24:1, v/v) and finally the supernatant was mixed with equal volume of isopropyl alcohol and incubated at −20°C for 30 min. After centrifugation, the pellet was dried, washed twice with 70% ethanol. The DNA pellet was air-dried and dissolved in 50 µL of TE buffer. All centrifugations were carried out at 12,000 rpm at RT for 10 min. The quality of the genomic DNA was checked by gel electrophoresis in 1% agarose gel (w/v). DNA quantification was checked by Nanodrop UV spectrophotometer.

### 2.3 PCR amplification conditions

Each PCR reaction was performed in a total volume of 20 µL containing 10× PCR buffer, 0.3 mM $MgCl_2$, 0.1 mM dNTPs (0.025 mM each dNTP), 1.8 ng/µL ISSR primer (Table 2), 0.04 U/µL Taq DNA polymerase (New England Biolab) and 20 ng/µL of genomic DNA. The PCR amplification program was set up an initial denaturation at 94°C for 5 min followed by 40 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 45°C for 30 s and extension at 72°C for 1 min. It was followed by a final extension at 72°C for 7 min. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel in 1× TAE buffer at 60V together with 2-Log ladder (NEB, England) to determine the molecular sizes of the fragments. After electrophoresis, gel was stained in 10 mg/L of ethidium bromide. Gels were photographed in a UV transillumunator of a Gel-Documentation System (Biorad).

**Table 2: List of primers were used in this study**

| STT | Primer code | Primer sequence |
|---|---|---|
| 1 | ISSR1 | 5' YAY GYA CAY $(TG)_7$ T 3' |
| 2 | ISSR2 | 5' RAY RAT AY $(GA)_7$ 3' |
| 3 | ISSR3 | 5' YGY RAY $(GA)_8$ 3' |
| 4 | ISSR4 | 5' (GA)8 RGY 3' |
| 5 | ISSR5 | 5' CRT AY $(GT)_9$ 3' |
| 6 | ISSR6 | 5' YGR GY $(GCC)_4$ 3' |
| 7 | ISSR7 | 5' ARR TY $(CAG)_4$ 3' |
| 8 | ISSR8 | 5' RYR CY $(AAT)_4$ 3' |
| 9 | ISSR9 | 5' RA TYT $(ATT)_4$ 3' |
| 10 | ISSR10 | 5' GAR TY $(ATT)_4$ 3' |
| 11 | ISSR11 | 5' CAC GTA CAC $(TG)_7$ T 3' |
| 12 | ISSR12 | 5' GAC GAT AT $(GA)_7$ 3' |
| 13 | ISSR17 | 5' CGT AAT $(GA)_7$ 3' |
| 14 | ISSR18 | 5' AGG TC $(CAG)_4$ 3' |
| 15 | ISSR3M | 5' $(CA)_6$ AG 3' |
| 16 | ISSR5M | 5' TCC TCC TCC TCC TCC 3' |

### 2.4 Similarity and dendrogram analysis

The bands were scored as 1 or 0 for the presence or absence respectively and similarity index were calculated from the data generated by using Jaccard's similarity index co-efficient and a dendrogram was constructed on the basis of the similarity matrix data by UPGMA clustering using the STATISTICA ver. 5.5 (StatSoft, 2000).

## 3 RESULTS AND DISCUSSION

The ISSR markers provide sufficient polymorphisms for characterization of varieties and genotypes of 16 chili rootstocks. 15 primers out of 16 primers were generated total of 136 distinct bands, with average of 9.06 scored able bands per primer in which 102 were considered as polymorphic. The percentage of poly-morphic loci was 83.3% indicating a higher level of polymorphism (Table 3). Among 15 primers, ISSR3M (Fig. 1) produced maximum number of polymorphic bands that indicated a high level of polymorphism as opposed to that of the primer ISSR5 (Fig. 1) generated the least number of polymorphic bands (Table 3). Evaluation of reproducibility of the banding pattern was confirmed by three replicated reactions with the same primer per chili sample.

**Table 3: 15 ISSR primers with corresponding bands score with polymorphic bands observed in 16 chili varieties**

| $N_o$ | Primers | Total number of bands scored | Number of polymorphic bands | Proportion of polymorphic loci (%) | The position of polymorphic loci |
|---|---|---|---|---|---|
| 1 | ISSR1 | 10 | 9 | 90.0 | 1,2,3,4,5,6,7,9,10 |
| 2 | ISSR2 | 14 | 10 | 71.4 | 1,2,4,6,7,8,11,12,13,14 |
| 3 | ISSR3 | 8 | 5 | 62.5 | 1,3,5,6,8 |
| 4 | ISSR4 | 9 | 7 | 77.8 | 1,2,3,5,6,8,9 |
| 5 | ISSR5 | 4 | 3 | 75.0 | 1,2,4 |
| 6 | ISSR6 | 9 | 6 | 66.7 | 1,4,5,6,7,9 |
| 7 | ISSR7 | 6 | 5 | 83.3 | 1,2,3,5,6 |
| 8 | ISSR9 | 9 | 5 | 55.6 | 1,2,3,4,6 |
| 9 | ISSR10 | 14 | 9 | 64.3 | 1,3,6,9,10,11,12,13,14 |
| 10 | ISSR11 | 10 | 6 | 60.0 | 1,2,3,4,5,6,9 |
| 11 | ISSR12 | 7 | 5 | 71.4 | 1,2,4,5,6 |
| 12 | ISSR17 | 11 | 11 | 100 | 1,2,3,4,5,6,7,8,9,10,11 |
| 13 | ISSR18 | 7 | 5 | 71.4 | 2,3,4,6,7 |
| 14 | ISSR3 M | 10 | 10 | 100 | 1,2,3,4,5,6,7,8,9,10 |
| 15 | ISSR5 M | 8 | 6 | 75.0 | 1,3,4,5,7,8 |
| | Total | 136 | 102 | | |
| | Average | 9.06 ± 2.69 | 7.29 ± 2.4 | 83.3 ± 16.0 | |

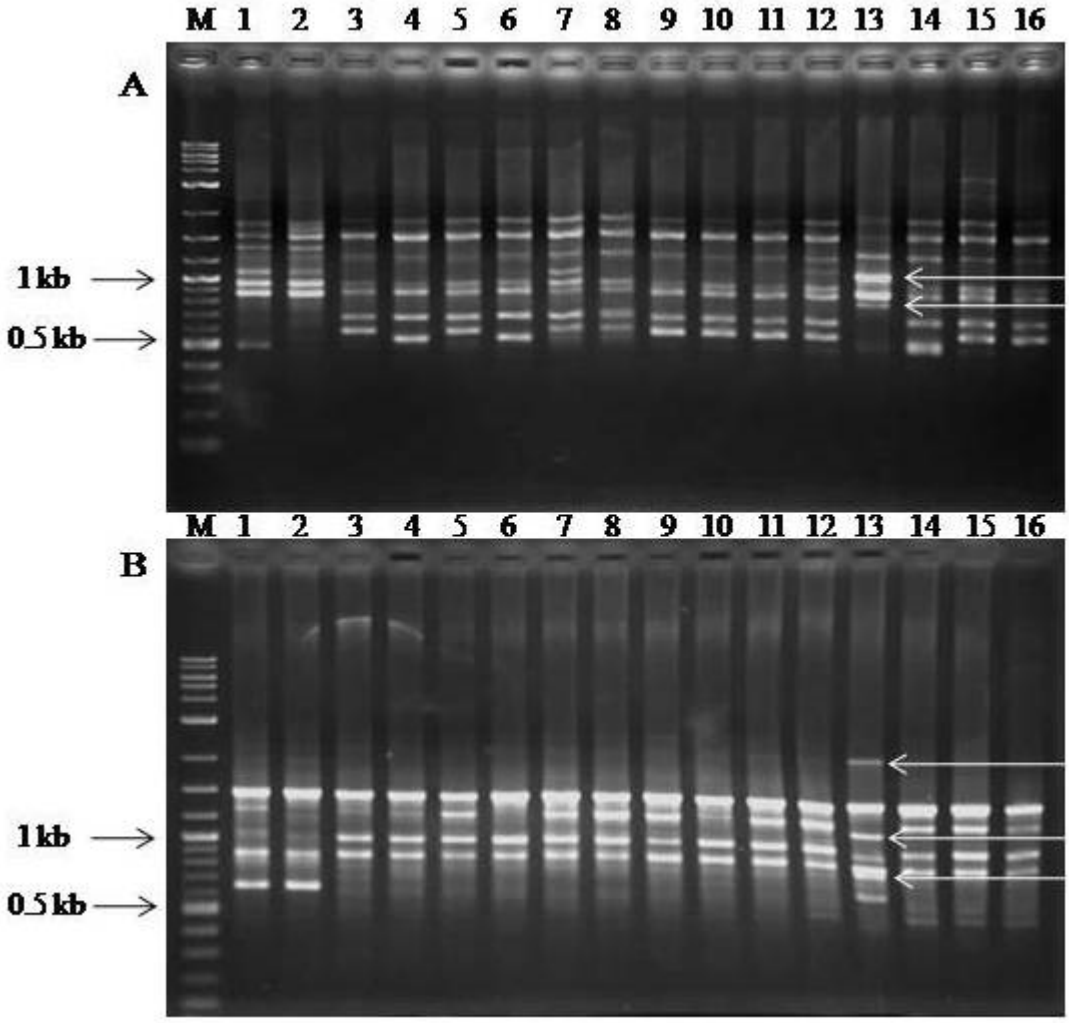


**Fig. 1: ISSR profiles from 16 chili varieties using ISSR3M (A) and ISSR5 (B) in which white arrow indicated polymorphic bands. M: 2-log ladder (NEB), 1. Hiem lai 207, 2. Sung vang Chau phi, 3. Ot Hiem trang, 4. Ot Hiem xanh, 5. Ot TN588, 6. Ot TN589, 7. Ot TN591, 8. Ot TN592, 9. Ot TN596, 10. Ot TN557, 11. Ot TN598, 12.Ot TN607, 13. Ot TN608, 14. Hiem 01, 15. Ot Da Lat, 16. Ot Hiem 27**

The genetic similarity among 16 rootstock chili varieties was generated based on the combination of polymorphic bands from 15 primers ranged 2.00- 7.81 (Table 4). The genetic distance value between variety 1 and 13 was the highest (7.81), while the genetic distance between variety 9 and 10 was the lowest (2.00). This study indicated that the variety 1 and 10 showed the highest genetic variation, while the lowest genetic variation was observed between variety 9 and 10, the two latter cultivars can be used as parental source for breeding line to improve chili varieties. Moonmoon (2006) reported that assessment of genetic diversity, molecular markers were superior to morphological, biochemical and other methods like pedigree and heterosis.

**Table 4: Summary of similarity matrix of genetic distance values between 16 chili varieties**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 | | | | | | | | | | | | | | | |
| 2 | 5.66 | 0.00 | | | | | | | | | | | | | | |
| 3 | 6.48 | 6.00 | 0.00 | | | | | | | | | | | | | |
| 4 | 6.08 | 5.57 | 3.32 | 0.00 | | | | | | | | | | | | |
| 5 | 6.48 | 6.00 | 3.74 | 3.61 | 0.00 | | | | | | | | | | | |
| 6 | 6.48 | 5.48 | 4.24 | 3.32 | 3.74 | 0.00 | | | | | | | | | | |
| 7 | 6.40 | 5.39 | 3.61 | 3.16 | 3.00 | 3.00 | 0.00 | | | | | | | | | |
| 8 | 6.63 | 5.83 | 3.74 | 3.61 | 3.16 | 2.83 | 2.65 | 0.00 | | | | | | | | |
| 9 | 6.32 | 5.29 | 3.46 | 3.61 | 3.16 | 3.46 | 2.65 | 3.16 | 0.00 | | | | | | | |
| 10 | 6.63 | 5.66 | 3.16 | 3.61 | 3.46 | 3.46 | 3.00 | 2.83 | 2.00 | 0.00 | | | | | | |
| 11 | 6.32 | 5.48 | 3.74 | 3.87 | 3.74 | 3.46 | 3.32 | 3.46 | 2.45 | 2.83 | 0.00 | | | | | |
| 12 | 6.93 | 5.83 | 4.00 | 4.12 | 3.74 | 3.16 | 3.00 | 3.16 | 3.16 | 3.16 | 3.46 | 0.00 | | | | |
| 13 | 7.81 | 7.14 | 7.42 | 7.35 | 7.28 | 7.14 | 6.93 | 7.14 | 7.28 | 7.28 | 7.28 | 7.28 | 0.00 | | | |
| 14 | 6.86 | 6.24 | 4.36 | 4.24 | 4.36 | 4.12 | 4.24 | 4.36 | 4.12 | 4.12 | 4.58 | 4.36 | 7.62 | 0.00 | | |
| 15 | 7.00 | 6.40 | 4.12 | 4.24 | 4.12 | 4.36 | 3.74 | 4.12 | 3.87 | 3.87 | 4.12 | 3.87 | 7.48 | 4.24 | 0.00 | |
| 16 | 6.78 | 6.16 | 4.00 | 3.87 | 4.00 | 3.74 | 3.32 | 3.74 | 3.46 | 3.46 | 4.00 | 3.74 | 7.28 | 4.12 | 3.32 | 0.00 |

*Note: 1. Hiem lai 207, 2. Sung vang Châu phi, 3. Ot Hiem trắng, 4. Ot Hiem xanh, 5. Ot TN588, 6. Ot TN589, 7. Ot TN591, 8. Ot TN592, 9. Ot TN596, 10. Ot TN557, 11. Ot TN598, 12.Ot TN607, 13. Ot TN608, 14. Hiem 01, 15. Ot Đà lạt, 16. Ot Hiem 27*

Similarity matrix was used for UPGMA cluster analysis to generate a dendrogram of 16 chili varieties (Fig. 2). According to the dendrogram, 16 chili varieties were classified into 5 groups showing a complex relationship between the genotypes. In the cluster analysis, five main clusters could be identified. The first group was identified as Hiem Lai 207, a scion with small fruit size and highest fruit number. The second group was Ot Sung Vang Chau Phi using as scion, a commercial variety with highest yield. For instance, genotypes Ot Hiem Xanh, Ot Hiem Trang, Ot TN588, Ot TN589, Ot TN591, Ot TN592, Ot TN596, Ot TN557, Ot TN598, Ot TN607, Ot Da lat, Ot Hiem 27 were clustered in the same group with genetically distinct from other chilies examined. In addition, the group 4 and 5, Ot Hiem 01 and Ot TN 608 were the most infected from pest at early stages, neither of them could not be used for planted in Mekong delta. These assessments of genetic variation among the chili genotypes provided defines a marker array for germplasm collection.

Results of ISSR profiles (Fig. 2) have been identified species-specific bands for hybrids individually, which could be used as molecular markers for hybrid chillies. These findings were agreed with those finding from Wang and Fan (1998) and Patel *et al*. (2011) regarding the high degree of polymorphism among Capsicum germplasm through ISSR markers. The values of similarity coefficient and the UPGMA dendrogram showed narrow genetic base among the tested hybrids in examine samples. The result is likely agreement with the fact that their parental breeding lines used to develop these hybrids were very close to each other, bearing in mind that pepper is a self-pollinated crop. This study confirmed the results from other research groups such as Kumar *et al*. (2001); Ilbi (2003); Yang *et al*. (2005); Akbar *et al*. (2010) and Lijun and Xuexiao (2012) who reported that the genetic variations of Capsicum species can be used DNA based data for studying the phylogenetic relationship among various accessions of a species based on geographic origin. Thus, ISSR technique has been successfully used to differentiate and the cultivars within the *Capsicum* species, which is useful in germplasm conservation and breeding programs.

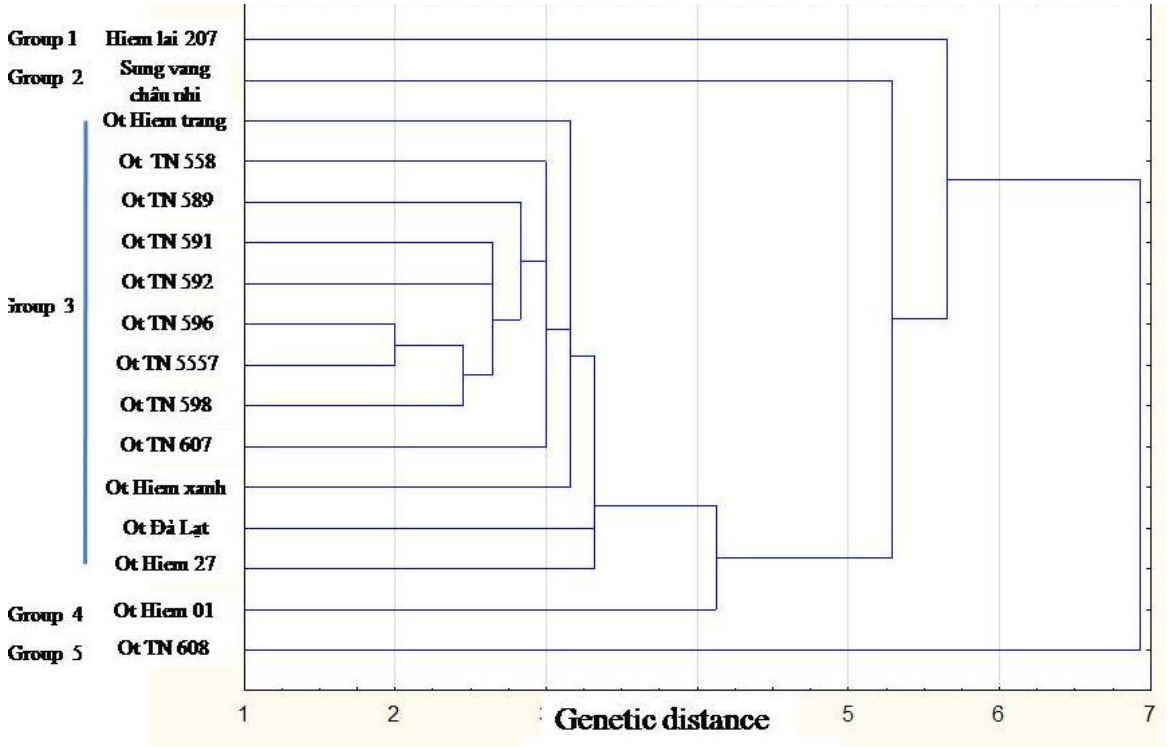


**Fig. 2: Dendrogram of 16 chili rootstock varieties using 15 ISSR analysis data from a the Jaccard's similarity matrix and UPGMA method**

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