Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE MOLECULAR ASSESSMENT OF GENETIC DIVERSITY OF EGGPLANT CULTIVARS FROM NORTHERN KARNATAKA IN INDIA USING RANDOMLY AMPLIFIED POLYMORPHIC DNA MARKERS

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Abstract

This is the most comprehensive study to present the genetic diversity available in populations of eggplant cultivars using molecular markers. An attempt was made to analyze genetic variability among twelve eggplant cultivars by Randomly Amplified Polymorphic DNA (RAPD) techniques using twenty random primers. Among the twenty RAPD primers used, OPA-04, OPA-07, OPA-14 and OPA-20 primers gave the best positive results with all twelve egg plant cultivars and bands generated showed a higher level of polymorphism. This reveals a very narrow genetic base of the different collections and the results also indicate that all the populations show more than 80% similarity irrespective of their flower colour and other morphological features. POPGENE software was used for the population genetic analysis, each band produced was treated as a locus and variations among the alleles were calculated. The RAPD markers used in the present study were able to differentiate the genetic diversity in the eggplant cultivars. The genetic diversity of eggplant cultivars was revealed by percentage of polymorphic loci. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated in the present study shows the segregation of twelve cultivars of eggplant into three main clusters: five entries were grouped into cluster 1, four entries were grouped into cluster 2 and remaining three entries were grouped into cluster 3. The results of RAPD showed that there appears a need to take individuals from more different populations so as to preserve their diversity for the future. The study also confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different accessions of an important vegetable plant like eggplant.

Key words*: eggplant, genetic variability, RAPD, northern karnataka, molecular markers.*

INTRODUCTION

Biodiversity may be defined as the variation present in all species of all life forms that exist in nature, their genetic material and the ecosystems in which they occur. Diversity can occur at three levels: genetic diversity, species diversity and ecosystem diversity. The importance of biodiversity for human beings has been well recognized in the recent decades and many would argue that diversity is essential for allowing sustainable development of various human activities. Biodiversity enables us to develop social and economic systems that allow the poorest to fulfil their food and nutritional needs and retain the ethnic diversity of countries all over the world (Shiva, 1994). The bioresources of each nation are important for the sustainable development of that nation, but only few countries are endowed, and coordination among countries is required for successful conservation and sustainable use of our biodiversity.

Genetic diversity or variation is important in the process of plant and is also basis of genetic fingerprinting (Sakata et al., 1991; Perez et al., 1998; Larisa, 2008). Assessment of the extent and distribution of genetic variation in a particular plant species along with its relatives is essential in understanding pattern of diversity and evolutionary relationships between accession that help to sample genetic resources in a more systematic fashion for conservation and improvement (Halldén et al., 1996; Patil et al., 2007).

Molecular markers are indispensable tools for understanding the genetic make-up of agricultural and horticultural crops. Molecular

markers allow researchers and molecular plant breeders to study and analyse genetic diversity or variation between two or more plant genomes. Molecular markers are similar to genetic markers and have received much attention these days (Haley et al., 1993; Haig et al., 1994; Larson et al., 1996). Genetic markers are seen as morphological differences. Since from the past three decades, phenotypic differences have been used to build genetic maps. Molecular markers differ from genetic markers in several ways: (1) molecular markers usually occur in greater numbers; (2) molecular markers can be distinguished without depending on complete growth of the plant that is tissue from a plantlet may be analysed rather than waiting for the plant to exhibit some morphological features; and (3) the environment does not alter the expression of molecular markers (Mullis et al., 1986; Hadrys et al., 1992; Prakash et al., 2006; Singh et al., 2006).

Generally, molecular markers are used for the genetic diversity, systematic and phylogenetic analysis. They are used in combination with other markers to construct genetic maps and gene factor genetic linkage studies (Bosland and Votava, 2000; Anderson, 2007; Bosland et al., 2012). Marker linked to a desired trait can be used by plant breeders in marker-assisted selection of qualitative and quantitative traits. When specific markers are identified with gene of interest and these microsatellite markers can be utilized as a selection criterion by molecular plant breeders and selection via marker-assisted selection speeds up the process of new variety development.

Cultivated plants have the market value and consumer preference (Sidhu et al., 2008). These plants perform in good sense against diseases, insects, nematodes and herbicides. These cultivated plants have good nutritive value when compared to the wild one. Present days many hybrid plants have more edible value and hence, every farmer concern about their crop, consumer need and marketing value. Now, consumer prefers hybrid varieties for their food because of different taste. The hybrids also have above mentioned good values when compared to its wild varieties (Nassar, 2000). Many of the breeders or farmers are growing hybrid varieties.

Brinjal eggplant (*Solanum melongena* L.) is an agronomically important crop of Solanaceae family, native to India, and widely cultivated across the globe. Its close relatives are *S. aethiopicum* and *S. macrocarpon* are of African origin. Karihaloo and Gottlieb (1995) proved genetic similarity between *S. melongena, S. incanum* (wild form) and *S. insanum* with allozyme studies and on the basis of the random amplified polymorphic DNA (RAPD) variation (Karihaloo et al., 1995). Much more studies have been done for disease resistance and abiotic stress by using RAPD markers (Doganlar et al., 2002; Frary et al., 2003; Sekara et al., 2007; Prabhu et al., 2009; Tümbilen et al., 2011; Sharmin et al., 2011; Laila et al., 2012; Manjusha et al., 2012; Singh and Malik, 2012; Mohd et al., 2013; Ramesh et al., 2013; Sifau et al., 2014). In this context, the paper presents an analysis of genetic diversity of eggplant cultivars using different RAPD markers.

MATERIALS AND METHODS

Plant Material

The present investigations were carried out at Department of Botany of Karnatak University in Dharwad, Karnataka. The experimental material consists of twelve hybrids of eggplant (Table 1).

Table 1. List of eggplant cultivars used in the present study

Hybrid seeds	Abbreviation
Mahya MEBH-11	MB
Kuduchi-601	KU
Syngenta EPH 718	SY
Sarpan SBH-65	SS65
Sarpan-25	S25
Ruchica	RU
Sarpan Green long	SGL
Sarpan Purple long	SPL
Simisi manjra	SM
Sarpan SBH-55	SS55
Green oval	GO
Ramya	RA

The hybrid seeds were sown in raised nursery bed and covered by the thin layer of sand and watered every day. Hybrid seeds were collected from Sarpan Hybrid Seeds Co. Pvt. Ltd., Dharwad and from farmers' fields in Kalagatagi, Jamakandi, and Belgaum. These are popularly eggplant growing areas of northern Karnataka.

Reagent for DNA isolation

200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS; the proper mixture of these solutions is called as Edwards Reagents.

Reagent for Agarose electrophoresis gel

Agarose; TAE buffer Stock (50X) - 1000ml (Tris base – 242 g, Glacial acetic acid – 57.1ml) - from this stock 0.5X is taken and made up to 2500 ml for running buffer; Blue loading dye; Ethidium Bromide solution - >10mg/ml.

DNA Isolation

For DNA isolation quick extraction method was used instead of CTAB method because of less period of time. This method includes Edward DNA isolation solution. For DNA extraction, Edwards solution was diluted to 10-fold to obtain extraction buffer. A 40 mg of leaf sample was weighed. The leaf sample was chopped to fine pieces with scissors and clean scalpel blade and fine leaf pieces were placed in an Eppendorf tube. About 400µl of DNA extraction buffer was added to the tube. The leaf tissue is grinded with a plastic rod against the tube wall. Once the solution turns to transparent green, the solution is centrifuged at 10000 rpm for 6min. The pellet was discarded and supernatant was stored at -20^{o}C. The collected supernatant is a source of DNA template for further experiments (Edwards, 1991; Lickfeldt et al., 2002; Amani et al., 2011). The quantification of DNA was done by the Agarose gel electrophoresis and Nanodrop method.

Agarose gel electrophoresis

Following the treatment of DNA samples, the electrophoresis of the samples were done according to the Sambrook procedure (Sambrook et al., 1989): 250mg of agarose was weighed and dissolved in 25 ml of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1 liter) and boiled in microwave oven. The mixture was allowed to attain 55^{o}C, and then poured it into the gel cassette fitted with comb. Allow the gel to solidify and carefully comb was removed, later the gel was placed in the electrophoresis chamber flooded with TAE buffer. 10 µL of DNA sample (mixed with bromophenol blue dye @ 1:1 ratio) was loaded carefully into the wells, along with standard DNA marker and 50 V of electricity for around 45 min was passed. After the gel was eluted with ETBR solution (10 µL/ml) for 10-15 min and bands were observed under UV transilluminator and confirmed the DNA in sample.

DNA Quantification by Nanodrop technique

For PCR amplification, the concentration and purity of DNA is a primary requisite. Different DNA extraction procedures yield DNA of different quantity and quality, for higher resolution and better reproducibility it is necessary to optimize the concentration of DNA. RAPD amplification is no longer reproducible when certain critical concentration of genomic DNA is less than minimum quantity (Williams et al., 1990; Fraga et al., 2005). Hence, it is necessary to stay above this critical concentration. Moreover, excessive DNA concentration is likely to produce poor resolution or “smear” resulting in a lack of clearly defined bands in the gel. However It is the always best to carry out a series of RAPD reactions using a multiple set of primers and a set of serial dilutions of particular genomic DNA to verify the critical DNA concentrations for better reproducibility of RAPD pattern.

Amplification of RAPD markers by Polymerase Chain Reaction (PCR)

Twenty OPA decamer primers of random sequence were screened initially on a sub sample of four randomly chosen individuals to evaluate their suitability for DNA amplification, which could be accurately scored (Easmin et al., 2008). Primers suitability was evaluated based on the resolution of bands, repeatability of markers and compatibility and comparability within individuals and among varieties (polymorphism). The optimal template concentration was not identical for all primers and furthermore, not all primers performed equally well. Some, presumably due to lack of suitable priming sites in the genomic DNA,

gave poorly binding patterns, while other create polymorphic bands, even within a set of identical replicated DNA samples. Thus, oligo-nucleotides should be rigorously tested for priming ability and reproducibility before using as markers. For primer selection, PCR reactions were performed on each DNA sample in a 20 µl reaction mix containing the following reagents: Master Mix (10.0 µl), primer (1.0 µl), DNA sample (1.0 µl) and Nanopure water (8.0 µl).

Master Mix includes the composition as Ampli Taq polymerase buffer (l0X), dNTPs and Ampli Taq DNA polymerase. For electrophoresis, 2 µl loading dye was added in the reaction mix to make total volume 22 µL. To perform Polymerase Chain Reaction (PCR) Reaction mix preparation and conditions for RAPD amplification reactions were followed according to Williams et al. (1990) with slight modifications. PCR reactions were performed on each DNA sample using a 20 µl PCR reaction mix as mentioned elsewhere. During the experiment, Master Mix and primer solutions were thawed from frozen stocks, mixed by vortexing and placed on ice. DNA samples were allowed to attain room temperature and mixed gently. The primers were pipetted into PCR tubes (0.2 ml). For each DNA sample, a pre-mix was then prepared in the following order: Master Mix, DNA template and Nanopure water. The tubes were sealed and placed in thermocycler for amplification.

DNA amplification was performed accordingly as follows: preheating is 95°C for 5 min., 45 cycles of denaturation is 95°C for 1 min., annealing is 95°C for 2 min., elongation is 72°C for 2 min. then the final elongation is 72°C for 10 min. After completion of cycling program, reactions were held at 4°C and gel electrophoresis was performed.

Since RAPD markers are dominant in nature, each band represents the phenotype at a single allelic locus (Williams et al., 1990). One molecular weight marker, 1 kb DNA ladder was used to estimate the size of the amplification products by comparing the distance travelled by each fragment with that of the known fragments of molecular weight markers. All distinct bands (RAPD markers) were allotted with identical numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. Bands not identified by the readers were considered as non-scorable.

A single data matrix was created by summing scores of all primers used in RAPD analysis and the same was used to estimate polymorphic loci, gene diversity, population differentiation, gene flow, genetic distance and to construct UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program, POPGENE (Version 1.31) (Nei, 1973; Yeh et al., 1999; Ghose et al., 2014). The same program was also used to perform test of homogeneity in different loci between population pairs.

RESULTS AND DISCUSSIONS

RAPD can be considered to be essential tool in cultivar identification (DNA typing), assessment of genetic variability and relationship management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping (Hasibe, 2009). An attempt was made to analyse genetic variability among eggplant cultivars by Randomly Amplified Polymorphic DNA (RAPD) technique. We used 20 different RAPD primers (Table 2) for the present study. Among the 20 primers initially tested all primers with a single variety of DNA. It gives yield as comparatively maximum number of amplification products with high intensity, with minimal smearing, good resolution and also clear bands. The number of fragments amplified per primer varied. In case of 12 eggplant cultivars out of 20 OPA primers only four primers generate bands, which shows proliferation of DNA and these particular primers are binds with the DNA. OPA-04, OPA-06, OPA-07, OPA-08, OPA-14 and OPA-20 RAPD primers gave positive result with one variety of eggplant cultivar Syngenta but OPA-06 and OPA-08 primers shows very weak binding signals. After this result we selected four except OPA-06 and OPA-08 primers for further study, and perform four primers with the all eggplant cultivars and get maximum bands in OPA-07 (Figure 1) and OPA-14

(Figure 2). The generated bands show the highest numbers of polymorphic bands. Thus it showed a higher level of polymorphism. The four primers generated 5 to 10 scorable bands per primer and all bands shows polymorphic RAPD markers per primer but in OPA-14 one band shows monomorphic. Strong and weak bands were produced in the RAPD reactions. A weak band denotes low homology between the primer and the pairing site on the nucleotide sequence (Thormann et al., 1994).

Table 2. List of primers used for the RAPD analysis in the present study

Primers	Sequence	GC Content (%)
OPA-01	5'-CAGGCCCTTC-3'	70
OPA-02	5'-TGCCGAGCTG-3'	70
OPA-03	5'-AGTCAGCCAC-3'	60
OPA-04	5'-AATCGGGCTG-3'	60
OPA-05	5'-AGGGGTCTTG-3'	60
OPA-06	5'-GGTCCCTGAC-3'	70
OPA-07	5-GAAACGGGTG-3'	60
OPA-08	5'-GTGACGTAGG-3'	60
OPA-09	5'-GGGTAACGCC-3'	70
OPA-10	5'-GTGATCGCAG-3'	60
OPA-11	5'-CAATCGCCGT-3'	60
OPA-12	5'-TCGGCGATAG-3'	60
OPA-13	5'-CAGCACCCAC-3'	70
OPA-14	5'-TCTGTGCTGG-3'	60
OPA-15	5'-TTCCGAACCC-3'	60
OPA-16	5-AGCCAGCGAA-3'	60
OPA-17	5'-GACCAGCGAA-3'	60
OPA-18	5'-AGGTGACCGT-3'	60
OPA-19	5'-CAAACGTCGG-3'	60
OPA-20	5'-GTTGCGATCC-3'	60

Population genetic analysis in different eggplant cultivars was done using POPGENE software. The RAPD markers used in the study were able to differentiate the genetic diversity in the eggplant cultivars. The genetic diversity of eggplant was revealed by percentage of polymorphic loci. The genetic variations were analysed by the band presence *vs* band absence and this is due to incapability to prime a site in some strand difference or by deletions or insertions among priming sites on the DNA strand (Clark and Lanigan, 1993).

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the 12 cultivars of eggplant into three main clusters: five entries were grouped into cluster 1, four entries were grouped into cluster 2 and remaining three entries were grouped into cluster 3. In cluster 1, Sarpan brinjal–65 alone makes sub cluster 1 and Kuduchi-601, Mahya MEBH-11, Sarpan brinjal green long, Sarpan brinjal purple long make all together to form cluster (Figure 3). Genetic distance higher between Sarpan brinjal- 65 *vs* other varieties. In cluster 2, Simisi manjra, Syngenta, Ruchica, Ramya cultivars of eggplant have same genetic distance between them. In cluster 3, Sarpan brinjal green oval alone make sub cluster 1 in cluster 3, Sarpan brinjal-25, Sarpan brinjal SPH-55 have same genetic distance but Sarpan brinjal green oval have some distance from the both varieties which are there along with it. Sarpan brinjal SPH-55 shows consistency of its resistance character to diseases has been proved over the years based on its field performance (Sarpan Seeds Pvt. Ltd., Dharwad).

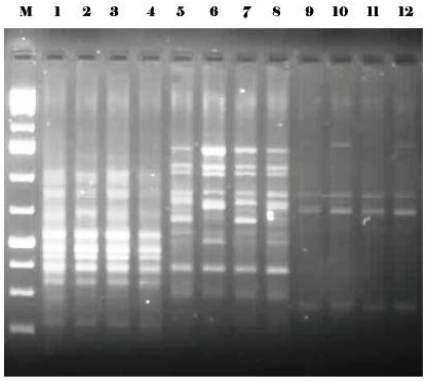

Figure 1. Different eggplant cultivar DNA Bands occur in gel electrophoresis with OPA-07

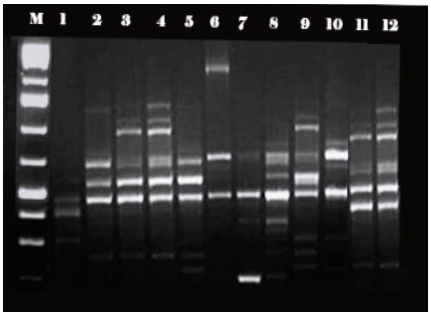

Figure 2. RAPD banding pattern obtained for twelve populations of eggplant with OPA-14

The RAPD analysis employed 20 random primers, four of which generated 20 polymorphic and 5 monomorphic markers with an average 2 to 4 bands per primer except OPA-07 it shows 7 to 9 DNA bands. Cluster

analysis of RAPD using POPGENE resulted into three clusters (Table 3). The present study reveals that among the PCR based fingerprinting techniques, RAPD is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationship between different cultivars of eggplant with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The information obtain from the present study could be of practical use for cultivar identification and validity as well as for classical and molecular breeding, as many of these cultivars are sources of vitamins and iron. The genetic similarity of among the cultivars is low as indicated by RAPD analysis. The informative primers identified in our studies will be useful in genetic and systematic analysis. The specific bands like OPA primers can be used as probes to certain eggplant cultivars they are in low or high copy numbers in the eggplant genome and such specific bands may be used for characterization and grouping eggplant cultivar. The study also provides a basis for crop breeders to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated with a cultivated species of eggplant. In a preliminary study, the genetic relationship between 12 populations, representative of the geographical distribution were investigated by the bulked genomic DNA-based methodology. This methodology has allowed the twelve populations for a detailed study of their genetic diversity. Most of these populations were indistinguishable from one another, but minimum genetic diversity among the 12 populations. The indistinguishable population was analysed further in this study to verify whether RAPD methodology using bulk DNA and individual samples give congruent results. Several primers were monomorphic with the bulk DNA procedure were revealed to be polymorphic with individual samples. Others, which were polymorphic with the bulk DNA were also polymorphic with individual populations, but were not retain in our study because the assignment of absence and presence of alleles was not clear for a number of sample. This can be explained by the fact that with bulk DNA, the alleles that are present in higher proportions are likely to be amplified with the random primers in such a way that rare alleles are not detected. Previous studies have shown that with the bulk DNA procedure, a polymorphic allele could be detected provided it represented at least 10% of the bulk (Paran et al., 1993).

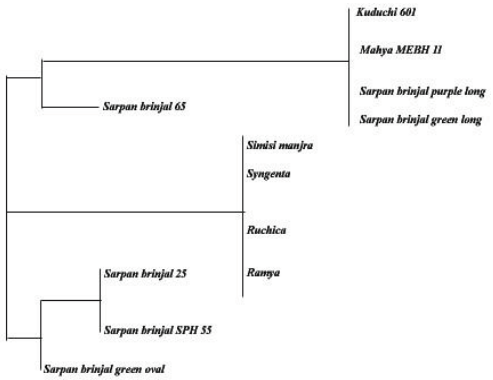

Figure 3. The differentiation between 12 eggplant genotypes based on RAPD analysis and Nei's (1972) genetic distance

Selection of polymorphic alleles was carried out very conservatively, and only clear and repeated polymorphic bands were selected. 14 out of 20 primers produced banding patterns that could be scored easily. The number of shared bands among individuals was evidence for the high degree of similarity among the populations (Table 4). The dendrogram constructed based on RAPD results obtained from four primers reflected the phenotypic variation observed in eggplant and related species during their collections. It is apparent from the dendrogram that collections originating from different places of the study area did not give well-defined distinctive clusters. They were interspersed with each other, indicating no association between RAPD pattern and the area of collection of accessions. This however, contrasted with the finding of Ge et al. (2013) who used SSR markers to get clusters among Chinese eggplant cultivers that resulted in clades subsequent to the geographic divisions.

Table 3. Scores of population genetic analysis in different eggplant cultivars; according to this DNA band data, dendrogram is drawn using POPGENE software

MB	1	1	1	1	1	0	0	0	0	0
KU	1	1	1	1	1	0	0	0	0	0
SY	1	1	1	1	1	0	0	0	0	0
SPL	1	1	1	1	1	0	0	0	0	0
SS55	0	1	0	0	0	1	1	1	1	1
SS65	0	1	0	0	1	0	1	1	1	1
S25	0	1	0	0	0	1	1	1	1	1
SGL	0	1	0	0	0	0	1	1	1	1
RU	0	0	0	0	0	0	1	1	0	0
GO	0	0	0	0	0	0	1	1	0	0
SM	0	0	0	0	0	0	1	1	0	0
RA	0	0	0	0	0	0	1	1	0	0

In Sarpan only many eggplant cultivars has showing wide spread genetic variability and Syngenta is close to the Mahya, Kuduchi and Sarpan purple long. Sarpan brinjal-65 have very wide genetic distance in cluster 1 based on the present RAPD analysis. Moreover, most of the eggplant cultivars shows low genetic variability when compared all other species, may be due to loss of alleles leads them to be low in number in the wild habitats needs further characterization and confirmation.

Morphological differences and similarities do not always reflect differences and similarity, because of genotype and environment interact-tion. As a result, the potential of making genetic progress is slow. In present study the Sarpan purple long and Sarpan green long are morphologically very similar and present in one cluster.

Therefore, in the future it is necessary to use DNA markers that will provide more rapid and precise information on the genetic diversity.

CONCLUSIONS

No molecular markers have been employed previously to quantify the genetic diversity within populations of eggplant cultivars in Northern Karnataka. This is the most comprehensive study to represent the genetic diversity available in populations of eggplant cultivars using molecular markers, which revealed a very narrow genetic base of the different collections. This study shows that if assay conditions are carefully controlled, the RAPD methodology may provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of populations and help devise sampling strategies to complement classical methodological agronomic descriptors. And the results also indicate that all the populations show more than 80% similarity irrespective of their flower colour and other morphological variability.

The results of RAPD showed that we need to take individuals from more different populations so as to preserve their diversity for the future. The study also confirms the suitability of RAPD as a rapid, cheaper, easy to handle and smart tool in molecular studies of different accessions in crop plants. Concurrently, it is also proved that the entries which were found to be similar in taxonomical classification based on morphological characters.

Table 4. RAPD bands generated by 4 primers in twelve cultivars of eggplant

Primer	Total number of bands	Total number of polymorphic bands	Number of polymorphic bands											
			A	B	C	D	E	F	G	H	I	J	K	L
OPA-04	3	2	2	0	2	0	2	0	1	1	2	2	2	2
OPA-07	10	10	9	8	8	9	8	9	8	8	1	2	1	2
OPA-14	8	6	2	3	4	6	3	2	1	4	6	2	4	6
OPA-20	4	2	2	2	2	2	2	2	2	2	2	2	2	2
Total	25	20	15	13	16	17	15	13	12	15	11	8	9	12
Polymorphism (%)			75	65	80	85	75	65	60	75	55	40	45	60

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