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ASSESSMENT OF GENETIC VARIABILITY AMONG *NICOTIANA RUSTICA* GENOTYPES BASED ON PRINCIPAL COMPONENT ANALYSIS AND CLUSTER ANALYSIS

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***N. rustica* is an important cultivated tobacco species that requires cooler climate and its cultivation is confined mainly to the northern and north eastern areas of the India i.e. Punjab, U.P. West Bengal, Bihar and Assam. Rustica tobacco are largely used for chewing, hookah and snuff purposes. ICAR-CTRI is maintaining a large rustica germplasm collection of about 800 accessions and is involved in developing improved rustica tobacco varieties. Genetic improvement of rustica depends mainly on the assessment of variability in the existing germplasm and its utilization. The genetic diversity of the existing rustica germplasm accessions were analysed based on 29 morphological parameters. Initially Principal component analysis (PCA) was done for the identification of morphological characters responsible for diversity. The PCA analysis showed that the genotypes were diverse in nature and the genotypes were scattered around the biplot. Out of 10 PCA components formed based on the eigen values, PCA1 accumulated 32 % of morphological variation loaded on inflorescence shape (IS), inflorescence compactness (IC), inflorescence position relative to upper leaves (IPRUL), flower length (FL), swelling of throat (FST), calyx nature (FCN), corolla shape (FCS), flower expression of tips of corolla (FETC), Flower Colour of Corolla (FCC), Flower Length of Pistil Relative to Stamens (FLPS) and development of stamens (FDS). Based on PCA1, PCA2 and PCA3 seventeen traits showing variation were selected for diversity analysis. The dendrogram showed the genotypes were grouped into four main clusters with many sub-clusters each. Genetic distance between clusters varies from 0.01 to 2.74. The results also showed that Goasani Goan-1, Mohark, M6, NC-64279, SK-49, SR-15 and SR- 29 were exhibiting more diversity when compared to other genotypes. Thus the study indicates that the germplasm was harbouring larger morphological variability which could be effectively used in the rustica tobacco breeding programme.**

INTRODUCTION

Tobacco is an important commercial crop grown in India providing higher farm returns to the farmers and fetching huge revenue to the exchequer. Out of over 75 naturally occurring *Nicotiana* species only two species i.e. *Nicotiana tabacum* and *Nicotiana rustica* are cultivated extensively. Two tobacco species viz., *Nicotiana tabaccum* and *N. rustica* are cultivated in India. Among the two, *N. rustica* requires cooler climate and hence, its cultivation is confined mainly to the northern and north eastern areas of the country i.e. Punjab, U.P. West Bengal, Bihar and Assam. Rustica tobacco are mainly used for chewing, hookah and snuff purposes. *N. rustica* has been evolved through the interspecific hybridization of *Nicotiana paniculata* and *N. undulata* about 200,000 years ago. *N rustica* has higher concentration of nicotine in its leaf (about 9%) when compared to *N tabaccum* (1-3%) (Katherine 2008). This higher concentration of nicotine is useful for pesticide industries and other fields across the world.

In India, ICAR-Central Tobacco Research Institute located at Rajahmundry takes the responsibility of crop improvement needs of *N.*

Key Words: Diversity, Germplasm, Rustica, Tobacco, Variability

rustica along with its germplasm. As a National Active Germplasm Site (NAGS) for tobacco germplasm, the institute is maintaining around 3300 tobacco accessions including 800 *N rustica* germplasms. The variability available in the *rustica* types maintained at the Institute was not studied till date. Studying the diversity of these accessions will helps in their effective utilization in breeding programmes aimed at higher yields, quality and resistance to stresses.

Diversity either natural or manmade is the primary force for crop evolution and genetic manipulation endeavors. The diversity in the biological system is the degree of difference between or within species and is explained in four different levels *viz*, ecological level, species level, genetic and genomic level. When the cultivated crop reaches the yield plateau, the variability available in the primary, secondary and tertiary gene pool becomes an inevitable source for breaking the yield barrier.

In the present study, an attempt was made to estimate the morphological diversity available in the rustica germplasm accessions maintained at the Institute. To measure the distance of any two genotypes cluster analysis was done to obtain the information on the trait values for dividing the population into various clusters. Additionally Principal Component analysis (PCA), a tool used frequently that identifies the component explaining the variability was also used. The main advantage of using PCA is it identifies the important traits that contribute to the variability. This method is being used worldwide in various crops like cotton (Kaleri *et al*, 2015), Soybean (Sreenivasa *et al*, 2019), rice (Prafull *et al*, 2015), wheat (Khodadadi *et al*, 2011) and finger-millet (Dosad *et al*, 2017). Hence, the current study was undertaken to visualize the morphological diversity present within *rustica* germplasm through cluster analysis and PCA approaches for its further utilization in genetic improvement programmes.

MATERIALS AND METHODS

Seedlings raised in the nursery from eight hundred rustica tobacco accessions maintained at the ICAR-CTRI gene bank during first week of October -mid November, 2018. Healthy seedlings thus obtained were transplanted to the main field in second fortnight of November 2018 at a spacing of 70 x 70 cm in a row trial. Recommended crop production and protection practices were followed to raise a healthy field crop. Observations were recorded on 29 morphological characters (Table 1) in three plants after confirming the uniformity within the row.

Statistical analysis: Initially, all the morphological observations were converted into scores (1 to 9) so as to resemble as qualitative characters as per the defined notes. The number of entries falling under different categories in each trait was compiled and their percentages were calculated. Morphological characters lacking variability were identified and excluded from further analysis. Principal component analysis (PCA) analysis was performed on the characters showing variability using SPSS 16.0 for the identification of morphological characters highly responsible for diversity. The resultant PCs with Eigen values greater than one were selected (Jeffers, 1967) for further analysis. The diversity prevailing among the *rustica* genotypes was computed using Computer Software Program–DARwin (Perrier and Jacquemond-Collet, 2006). Dissimilarly matrix for morphological observation was constructed using Rogers-Tanimoto coefficient of associations to find out genetic relationships among the accessions. These data were subjected to unweighted pair groups method with arithmetic mean (UPGMA) analysis to generate dendrogram using DARwin 5.0 and dissimilarity was estimated based on the respective morphological scoring.

RESULTS AND DISCUSSION

Twenty nine morphological observations were recorded in 800 *rustica* germplasms which included 5 plant characters, 11 leaf characters and 13 flower/testa characters (Table 1). The genotypes were found to have considerable variability for all the characters, especially the inflorescence traits recorded higher variation. Majority of the genotypes were cylindrical in shape (53%), very short statured (95%) and open in habit (55%). Such reduction in plant height is found when the crop is raised in tropical environment such as at Rajahmundry condition. Majority of accessions recorded few number (54%) of cordate (97%) leaves with well-defined petiole (100%). This is clearly differentiate

Table 1: Morphological observations recorded on the rustica entries

Character	Percentage plants under each category
Plant: shape	Conical (34.3), Cylindrical (52.9), Elliptical (12.9)
Plant: Height	Very Short (94.8), Short (4.9), Medium (0.4), Tall,Very (0), Tall (0)
Plant: Habit	Open (54.5), Erect (9.6), SemiErect (34.6), Squattering (0), Bouquet (1.3)
Plant: Internodal Length	< 4 cm (65.1),<6 cm (31.9), >6cm (3)
Plant: Number of Leaves	Few (54), Medium (46), Many (0)
Leaf: Type	Sessile (0), Petiolate (100)
Leaf: Angle of Insertion	Acute (8.4), Moderately Acute (77.2), Right Angle (14.4), Moderately Right (0)
Leaf: length	Very Short (23.8), Short (75.2), Medium (0.9), Long (0.1),Very Long (0)
Leaf: Width	Very Narrow (48.8), Narrow (48.9), Medium (2.3), Broad (0)
Leaf: Midrib	Thin (45.3), Medium (26.3), Thick (28.4)
Leaf: Blade Shape	Lanceolate (1.3), Narrow Elliptic (0), Broad lliptic (0), Ovate (1.3), Obovate (0.1), Cordate (97.4), Rounded (0)
Leaf: Tip Shape	Obtuse (94.1), Slightly Pointed (0), Medium Pointed (5.8), Strongly Pointed (0), Very Strongly Pointed (0.1)
Leaf: Blistering of Blade (puckering)	Absent or Very Weak (17.3),Weak (31.3), Medium (47.6),Strong (3.9),Very Strong (0)
Leaf: Undulations of Margin	Absent or Very Weak (31.4), Weak (29.6), Medium (36.3), Strong (2.8)
Leaf: Development of Auricles	Absent or Very Weak (98.8), Weak (0.9), Medium (0.4), Strong (0), Very Strong (0)
Leaf: Color of Blade	Yellow Green (0),White Green (0), Light Green (0.1), Medium Green (7.5), Dark Green (92.4)
Time of 50% Flowering (plants with at least one corolla open)	Very Early (56.1),Early (0), Medium (43.9), Late (0), Very Late (0)
Inflorescence: Shape	Spherical (28.2), Flattened Spherical (6.4), Inverted Conical (22.4), Double Conical (5.4), conical (28.2), cylindrical (9.4)
Inflorescence: Compactness	Very Loose (1.4), Loose / Slightly loose (11.1), Medium (42.9), Slightly Dense / Dence (44.3), Very Dence (0.4)
Inflorescence Position Relative to Upper Leaves	Among (6.5), Above (93.5)
Flower: Length /Size	Short (99.7), Medium (0.1), Long (0.2)
Flower: Swelling of throat	Small (83.0), Medium (16.6), Large (0.4)
Flower: Calyx nature	Equal (38.9), Unequal (61.1)
Flower: Corolla shape	Circular (24), Pentagonal (22.7), Obtusely Lobed (8.5), Acutely Lobed (36.8), Deeply Cleft (8.1)
Flower: Expression -Tips of Corolla	Absent (1.9), Weak (24.3), Medium (54.7), Strong (19.1),Very Strong (0)
Flower: Colour of Corolla	White (33.5), Greenish Yellow (59.9), Light Yellow (6.3), Yellow (0.3)
Flower: Length of Pistil Relative to Stamens	Shorter (23.4), Equal Length (45.2), Longer (31.5)
Flower: Development of Stamens	Absent (0.1), Full (99.9)
Testa: Colour	Dark Brown (99.3), Light Brown (0.8)

Note: values in the parenthesis indicates the percentage of genotypes expressing a trait

them from *N. tabaccum* accessions with majority of them having more number of sessile lanceolate to elliptic leaves (Sarala *et al.*, 2018 & 2019). Leaf Angle of insertion in majority of accessions is moderately acute (77%), length short (75%) and width narrow to very narrow (98%). The genotypes showed more variation for about 11 inflorescence characters. Majority of the accessions recorded very early time of flowering (56%), spherical and dence to medium inflorescence above the upper leaves. Unlike *N. tabaccum* flowers which are large and pinkish, *rustica* flowers are mostly small, yellowish and fertile. The testa colour of the seeds was dark brown (99%) in most of the entries. This is in contrast to the seeds of *N. tabaccum* accessions which in general have light brown testa colour (Sarala *et al.*, 2018 & 2019). It is observed that all the cultivated forms of *Nicotiana* come under same species except Rustica which forms separate clusters due to its variation in inflorescence characteristics (Goodspeed 1954; Chase *et al.* 2003; Knapp *et al.* 2004).

The variation contributed by all the 29 morphological characters to the diversity of the genotypes was studied for clustering the genotypes. The characters responsible for diversity of the genotypes were identified by Principle component analysis (PCA). Morphological observations were found to be explained by ten principal components accounting for about 73% of variability with PCA 1 capturing majority of the variability (Fig 1, Table 2). PCA1 accumulated 32 % of morphological variation and was loaded on Inflorescence Shape (IS), Inflorescence Compactness (IC), Inflorescence Position Relative to Upper Leaves (IPRUL), Flower Length (FL), Swelling of throat (FST), Calyx nature (FCN), corolla shape (FCS), Flower Expression of Tips of Corolla (FETC), Flower Colour of Corolla (FCC), Flower Length of Pistil Relative to Stamens (FLPS) and Development of Stamens (FDS). Sarala et al., (2018) in their study detected that PCA1 explained 26% of the variation loaded mainly on width of leaf base, plant leaf number, leaf length, leaf blade shape, leaf veins thickness and leaf development of auricles in mutant tobacco (*N. tabaccum*) germplasm accessions. Sarala et al.,

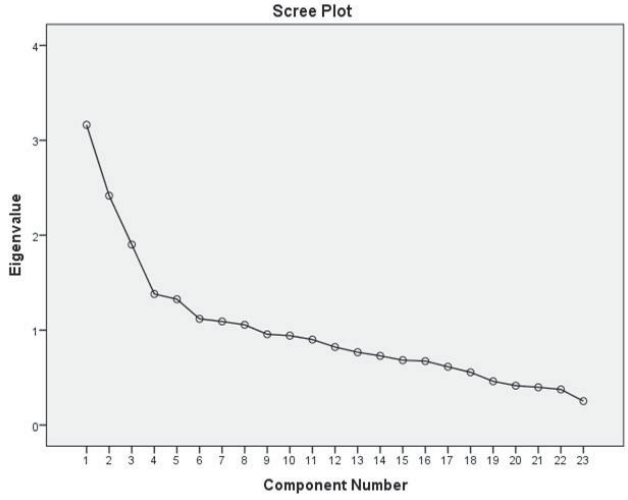

Fig. 1: Biplot explaining the total variability present in the rustica germplasm

Table 2: **Eigen values (Latent roots) and rotated component loadings (values of principal component traits of *rustica*)**

Component	Eigen value	Variability (%)	Cumulative
PCA1	9.285	32.019	32.019
PCA2	2.188	7.544	39.563
PCA3	1.599	5.513	45.076
PCA4	1.319	4.550	49.626
PCA5	1.221	4.212	53.837
PCA6	1.187	4.092	57.930
PCA7	1.123	3.874	61.804
PCA8	1.105	3.810	65.614
PCA9	1.062	3.662	69.276
PCA10	1.007	3.473	72.749

(2019) in another study with burley tobacco (*N. tabaccum*) observed that PCA1 explained 16% of the variation and was loaded mainly on eight traits viz., colour of leaf blade, leaf angle of insertion, inflorescence shape, leaf colour of mid-rib, width of leaf blade, plant shape, flower development of stamens and leaf length. The inflorescence characters showed high variability and they are expected to provide high level of gene transfer if used in breeding programs (Gana, 2006; Aliyu et al., 2000). The biplot shows that around 50 genotypes were varying highly from rest of the genotypes and these lines could be used in the breeding programme to exploit the unutilized variability (Fig. 2, Table 3). The vector length (i.e., the distance to the biplot origin) of a trait indicates how well the trait is represented in the biplot; a relatively short vector indicates that the variation of the trait across genotypes is either small or not well presented in the biplot, which is due to its weak or lack of correlation with other traits (Yan and Fregeau-Reid, 2018). Significant amount of difference in various morphological observations aids in grouping the genotypes into different clusters. Hence, based on PCA1, PCA2 and PCA3, one plant (number of leaves), four leaf (length, tip shape, blistering of blade and undulations of margin), three inflorescence (time of 50% flowering, inflorescence shape, compactness and position relative to upper leaves) and nine floral (flower length /size, swelling of throat, calyx nature, corolla shape, expression -tips of corolla, colour of corolla, length of pistil relative to stamens and development of stamens) characters were selected for further analysis as the remaining variables had weak or no discriminatory power (Fig. 3).

The dissimilarity index and relationship between the genotypes were further analyzed in DarWin 5.0 using the selected characters showing variability. The results also showed that Goasani

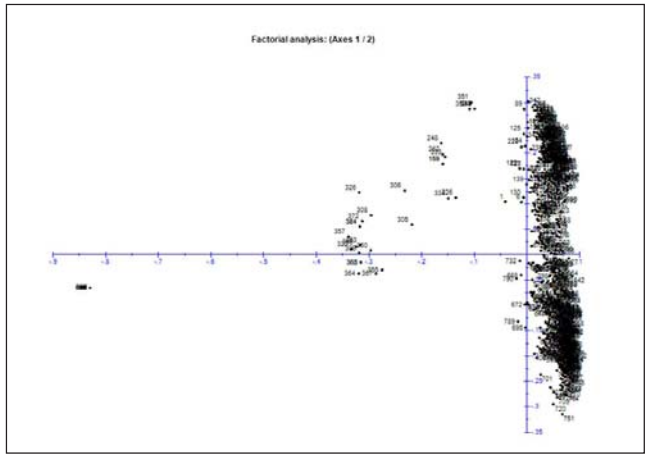

Fig. 2: Scatter diagram of biplot showing rustica genotypes based on PCA1, PCA2 and PCA3

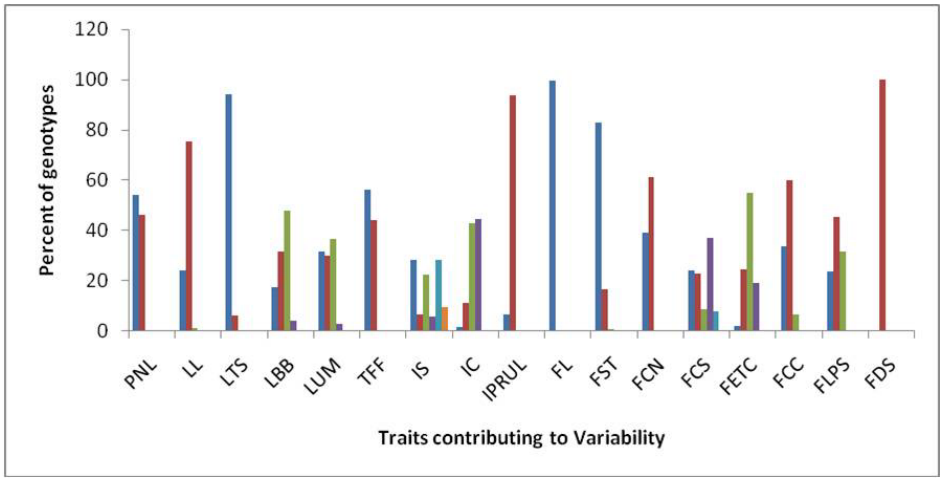

Fig. 3: Traits contributing to variability in rustica germplasm that are identified by PCA1, PCA2 and PCA3

Note: Each colour bar related to a variable in a trait. Please see the note given at Table 3 for 'X' axis legend details

Table 3: Eigen value ("Load") of the principal component matrix and its contribution to total variation of rustica entries

	Principal Component									
Character	**PCA1**	**PCA2**	**PCA3**	**PCA4**	**PCA5**	**PCA6**	**PCA7**	**PCA8**	**PCA9**	**PCA10**
PS	.039	.162	.070	-.218	-.485	-.139	-.144	.075	.533	-.328
PHT	-.051	-.050	.269	.057	.373	-.027	.599	-.187	.140	-.016
PH	-.002	.418	-.186	-.253	.176	-.046	-.130	-.168	.102	.135
PIL	-.101	-.313	.000	.159	-.074	.185	.511	-.011	.053	.167
PNL	-.110	-.181	**.587**	.025	.073	-.071	.069	.068	.372	-.016
LTH	-.009	.021	.293	.013	.117	.201	-.456	-.159	.105	.122
LAI	.141	.064	.215	.326	-.061	.540	-.176	-.311	-.060	.051
LL	-.241	-.141	**.672**	.034	.134	.085	-.116	.083	-.040	.050
LW	-.031	.279	-.289	.077	-.355	-.009	.284	-.413	.009	.073
LM	-.015	.201	.093	.017	.127	-.242	.060	.415	-.499	-.305
LBS	.033	.227	.332	-.423	-.229	.219	.138	-.099	-.369	.149
LTS	-.049	-.024	-.243	**.696**	.246	-.005	-.163	.164	.043	.010
LBB	-.004	**.698**	.274	-.082	.149	-.131	.052	-.025	-.135	.011
LUM	.136	**.759**	.206	.219	-.097	.170	.034	.103	.037	-.087
LDA	.034	.041	.102	-.026	-.187	-.233	.038	.452	.164	.711
LCB	.057	.259	.107	.041	.354	-.490	.021	-.349	.208	-.088
TFF	-.079	**.683**	-.063	.377	-.115	.081	.130	.168	.133	.116
IS	**.910**	-.100	.145	.149	-.185	-.155	.008	-.065	-.077	-.041
IC	**.910**	-.098	.149	.149	-.185	-.152	.006	-.067	-.078	-.042
IPRUL	**.887**	-.090	.145	.180	-.179	-.147	-.005	-.068	-.087	-.038
FL	**.910**	.077	-.108	-.134	.186	.140	.013	.071	.083	.041
FST	**.882**	.057	-.122	-.153	.206	.158	.025	.075	.086	.048
FCN	**.930**	-.082	.118	.125	-.146	-.120	.012	-.044	-.055	-.024
FCS	**.914**	.076	-.115	-.144	.188	.131	.003	.074	.084	.035
FETC	**.931**	.007	-.031	-.049	.087	.022	-.003	.016	.013	.048
FCC	**.942**	-.003	-.020	-.038	.082	.025	-.002	.012	.010	.049
FLPS	**.894**	.063	-.136	-.158	.195	.143	.003	.070	.086	.035
FDS	**.910**	-.085	.119	.123	-.150	-.125	.007	-.047	-.060	-.029
TC	.119	.000	.029	-.062	-.033	.387	.234	.344	.204	-.400

Note: Plant: shape (PS),Plant: Height (PHT), Plant: Habit (PH), Plant: Internodal Length (PIL), Plant: Number of Leaves (PNL), Leaf: Type (LTH), Leaf: Angle of Insertion (LAI), Leaf: length (LL), Leaf: Width (LW), Leaf: Midrib (LM), Leaf: Blade Shape (LBS), Leaf: Tip Shape (LTS), Leaf: Blistering of Blade (puckering) (LBB), Leaf: Undulations of Margin (LUM), Leaf: Development of Auricles (LDA), Leaf: Color of Blade (LCB), Time of 50% Flowering (TFF), Inflorescence: Shape (IS), Inflorescence: Compactness (IC), Inflorescence: Position Relative to Upper Leaves (IPRUL), Flower: Length /Size (FL), Flower: Swelling of throat (FST), Flower: Calyx nature (FCN), Flower: corolla shape (FCS), Flower: Expression -Tips of Corolla (FETC), Flower: Colour of Corolla (FCC), Flower: Length of Pistil Relative to Stamens (FLPS), Flower: Development of Stamens (FDS),Testa: Colour (TC)

Goan-1, Mohark, M6, NC-64279, SK-49, SR-15 and SR- 29 were exhibiting more diversity when compared to other genotypes. Clustering through unweighted pair groups produced grouping that defined four distinct clusters in 0 to 0.5 distance (Fig. 3). Each group was further divided into sub groups. There are too many sub-clusters observed in the study based on morphology, it is due to the genetic variability within tobacco genotypes is likely affected by several genetic bottlenecks (Lewis & Nicholson 2007). The entries under each group share some underlying biological relationship, and such associations can be useful for generating hypothesis for better understanding of knowledge

on the complex traits (Maji and Shaibu, 2012). Most of the genotypes were clustering in group 3 and group 4 making them largest groups. On the contrary, the group 1 and 2 were accommodating fewer genotypes.

Genetic distance which is the similarity between two genotypes needs to be analyzed before undertaking any crossing programme. More the genetic distance between the parents the heterosis is believed to be higher. In the present study, the entries namely AR-16 and AR-14 had the lowest genetic distance of about 0.001 and the genotypes II-281 and NR-834 had the maximum distance (26.07).

In general, the morphological classification has its own limitations and the morphological diversity analysis can be further substantiated with molecular studies to add more value to the data. The theoretically relatively low levels of genetic variability within tobacco germplasm pools needs to be investigated further (Garner *et al.* 1936; Murphy *et al.* 1987). The pathway of speciation in tobacco is the first limiting factor for minimal diversity within the genotypes. In many other earlier studies a high degree of genetic relatedness was found to exist among modern varieties in the different tobacco types (Garner *et al.* 1936; Murphy *et al.* 1987; Bindler *et al.* 2005). The reason for low diversity within tobacco varieties is mainly because, being quality oriented crop, the breeders are confined to use few genotypes in breeding programme (Baghyalakshmi *et al.*,2018). Additionally the information on genetic diversity available in germplasm and distinct traits contributing for more variation shall enable breeding of newer high yielding cultivars with economically useful traits (Santhy *et al.*, 2019).

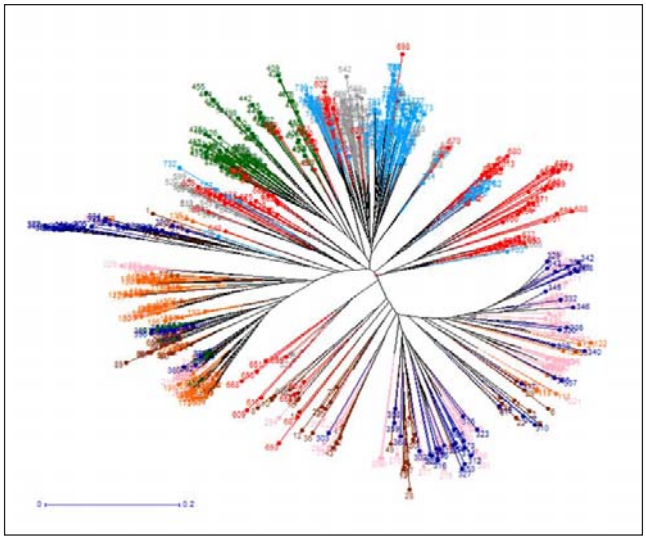

Fig. 4: Clustering pattern of the genotypes entries using DarWIN. Each genotype (line) is named with a prefix number.

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