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Joint analysis of qualitative and molecular diversity provides new insights on the genetic variability of the wild species of tomato (*Solanum section lycopersicum*) for quality attributes

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Abstract
The genetic variability of the tomato (*Solanum section lycopersicum*) 15 germplasms (seven different species) was assessed using a combination of qualitative and molecular data. The joint dissimilarity matrix showed moderate correlation with the original matrices of quality and molecular data. However, the correlation between the qualitative dissimilarity matrix and the genotypic dissimilarity matrix was low. This finding indicated that molecular markers (ISSR and SSR) did not adequately sample the genomic regions that were relevant for qualitative differentiation of the germplasm. The dissimilarity values of the joint dissimilarity matrix were used to measure qualitative + molecular diversity. This diversity varied from 0 to 1.43 among the 15 germplasm, with an average dissimilarity among genotypes of 0.62. Joint analysis of qualitative and molecular diversity indicated that the genetic diversity of the tomato germplasm was 174% and 69% higher than the diversity estimated from qualitative and molecular data, respectively. These results show that tomato genetic variability is not as limited as previously thought.

Keywords: qualitative variability, molecular variability, tomato, germplasm, dissimilarity matrix.

Introduction
Tomato (*Lycopersicon esculentum* Miller) is universally treated as 'Protective Food' since it is a rich source of minerals, vitamins and organic acids. The fruit is rich in lycopene, which may have beneficial health effects. The red pigment in tomato (lycopene) is now being considered as the "world's most powerful natural antioxidant" (Jones, 1999). Tomato is a very good appetizer and its soup is said to be a good remedy for patients suffering from constipation (Kalloo *et al*. 2001) [9]. Tomato cultivation has been increased since the mid-nineteenth century because of its varied climatic tolerance and high nutritive value. Tomato ranks third in priority after potato and onion in India but ranks second after potato in the world. It is grown both under field as well as in green house throughout the year. The major tomato growing countries are China, India, USA, Turkey, Egypt, Iran and Italy India ranks second in the area (0.88 Mha) with Production and Productivity of 18.73 MMT and 21.2 MT/ha, respectively in the world (Saxena *et al*., 2015) [10]. In Gujarat, tomato is grown in an area of 0.044 Mha with the annual production and productivity of 1.26 MMT and 28.2 MT/ha, respectively (Saxena *et al*., 2015) [10].

However, there is still a wide scope for increasing the inherent productivity potential of presently grown varieties. As a consequence of inbreeding during tomato domestication, the genetic diversity in cultivated tomato (*S. lycopersicum*) is very narrow (Foolad, 2007) [5]. The traditional cultivated tomatoes severely lack genetic diversity. Consequently, since ca. 1940, breeders have relied increasingly on exotic sources particularly related wild spp. for desired traits. Since then, accelerated introgression of useful exotic traits contributed to spectacular improvement, manifesting a four to five fold yield increase. Nearly unknown in tomato cultivars prior to 1940, resistance to at least 42 major diseases has been discovered in exotics and 20 of them transferred into cultivated tomatoes and the number is being continuously increasing (Vidavski *et al*., 2008) [13]. Based on the foregoing considerations, it is clear that the genetic variability of the tomato germplasm has not been fully investigated. Given the importance of this germplasm, it would be interesting not only to measure genetic diversity based on molecular or quality traits separately, but to combine the min to a single analysis in order to get a clear picture of the actual diversity available. Methods that allow such an analysis exist (Cruzet *et al*., 2011) [4], but are not used very often because only a few germplasm collections have been characterized qualitatively and molecularly.

Since the tomato germplasm collection has already been characterized qualitatively and molecularly, the objectives of this study were to (1) determine the extent to which molecular diversity resembles qualitative diversity, (2) determine how well the combined distance matrix correlates with distance matrices based on qualitative and molecular data, (3) evaluate the suitability of different procedures for performing a joint analysis of qualitative and molecular diversity, (4) verify whether a joint analysis of qualitative and molecular diversity could help discriminate genotypes within the tomato population and (5) verify whether a joint analysis of qualitative and molecular diversity can reveal novel patterns of genotype grouping. By using two complementary data sets (qualitative and molecular data previously generated by our group) we sought to provide a better assessment of the existing diversity. The results of this study may be helpful in determining the need for additional collections to improve the representativeness of the germplasm and allow further genetic characterization of tomato.

Material and Methods

Plant material and experimental design

The genetic variability of the 15 accessions including seven different species was assessed using a combination of quality and molecular data. The parents were selected from the breeding material maintained at farm of "Centre for Distant Hybridization in Field and Fruit Crops", Department of Agri-Biotechnology, Anand Agricultural University, Anand. The field experiment was done using a randomized blocks design with three replicates and ten adult plants per plot arranged in rows. Geographically, Anand (Agroclimatic Zone–III, Middle Gujarat) is situated at 22°35´ North latitude and 72°55´ East latitude with an altitude of 45.1 meters above the mean sea level. The soil of experimental site was sandy loam alluvial, deep, well-drained, moisture fair and responded well to manuring and irrigation.

Quality and molecular characterization

Quality evaluation of the 15 accessions including seven different species was done in the second year of cultivation and was based on reproductive traits. The following Quality traits were evaluated: (1) Moisture content (%), Anon.,1980, (2) Total Soluble Solids (° Brix), Zeiss Hand Refrectometer (3) Total Soluble Sugar (mg/100g), Phenol Sulphuric acid method (4) Reducing Sugar (mg/100g), Nelson's modification of Somogyi's method (5) Titratable Acidity (%), N/10 NaOH and Phenophthelin indicator method (6) Ascoric Acid (mg/100g), 2, 6-dichlorophenol indophenol titration method (7) Lycopene (mg/100g), Acetone-petroleum ether extraction method and (8)) Phenol (mg/100g), Folin ciocalteau method.

The molecular genomic DNA was extracted using the CTAB (Cetyltrimethyl Ammonium Bromide) method with some modifications (Murray and Thompson, 1980). The reagents and buffers for DNA isolation were prepared as per Sambrook *et al*. (1989). The DNA con-centration was measured using a Nanodrop spectrophoto-meter and the concentration of each sample was adjusted to 2-5 ng/ L. Seventeen ISSR primers and Sixteen SSR primer pairs were scored. The selected primers were previously tested for polymorphism in this same set of tomato accessions and found to amplify robust (reproducible) loci. ISSR and SSR loci were amplified by the polymerase chain reaction (PCR) using standard procedures. Bands amplified by total 17 ISSR and 16 EST-SSRs primers were used and primers were estimated on bases of their molecular weight of bands in base pairs; the clear and distinct bands amplified by markers were scored for the presence (1) and absence (0) of the corresponding band among the 15 tomato genotypes. Allele molecular weight (base pairs) was used to determine the genetic distance for phylogeny reconstruction using Neighbour Joining (NJ) method (Nei, 1973) with POWERMARKER. The scored bands were further analysed using NTSYS pc version 2.02 (Rohlf, 1998).

Genetic diversity analyses

Since we had access to Quality and molecular data, different strategies for studying the genetic diversity of tomato based on parallel molecular and Quality analyses were tested. Initially, the qualitative data were used to generate a dissimilarity matrix based on Gower's method (Gower, 1971) and the molecular data were used to generate a dissimilarity matrix based on Jaccard's coefficient (Jaccard, 1901). To combine quality and molecular data, the Gower and Jaccard dissimilarity matrices were summed algebraically to generate a new matrix referred to as the joint dissimilarity matrix. By combining dissimilarity matrices based on different data sets, *i.e.*, quality and molecular, our aim was to simultaneously assess all information at once, thereby capturing all available information.

Graphic representations of the matrices were generated based on colour gradients that were used to express the dissimilarity between genotypes. Red was used to indicate lower genetic dissimilarity while green was used to indicate the most dissimilar accessions. A score of zero corresponded to the lowest genetic dissimilarity (represented by red) while a score of 1 corresponded to maximum genetic dissimilarity (represented by green). The correlation between the dissimilarity matrices described above was also examined. The correlations and their significances were tested by using Mantel's Z test with 1000 permutations (Manly, 1997). This procedure allowed us to assess the resemblance between the genotypic and phenotypic matrices and between the genetic dissimilarity matrices and joint dissimilarity matrix.

After generating the dissimilarity matrices the genetic diversity of the tomato population was initially assessed based on a 3D dispersion analysis. For this, the dissimilarity matrices generated with Gower's index, Jaccard's index and by combining the Gower and Jaccard information were used to produce three groups of x, y and z coordinates, *i.e.*, a distance projection in three dimensional space using Genes software (Cruz, 2009). The coordinates were later used to construct 3D scatter plots with Sigma Plot v.12.0. In these graphs, greater dot dispersion indicated greater genetic diversity of the population. We later expanded this group analysis by using Tocher's clustering method. In this method the accessions were assigned to groups consisting of equivalent genotypes. The groups were presented using letter coded group profiles. Accessions allocated to the same group based on Tocher's cluster analysis were presented in the same colour in the 3D scatter plots.

Results

The quality and molecular diversity of tomato was assessed by generating various dissimilarity matrices based on Gower's and Jaccard's indices. Table 1 shows the minimum, maximum and mean genetic dissimilarities and Figure 1 shows the graphic representation of the dissimilarity matrices. The data in Table 1 and Figure 1 indicate the tomato diversity is limited at the molecular and quality levels, as shown by the yellow and red points in the figure. Red and yellow points indicate highly and moderately similar genotype pairs,

whereas green points indicate highly divergent genotype pairs. Although numerous red points have indicated low molecular diversity, the present data indicate that the mean molecular dissimilarity is greater than the mean quality dissimilarity. However, more genotypic pairs are closely related to each other based on molecular markers than on quality (Figure 1).

Table 1: Descriptive statistics for genetic diversity in the tomato according to the method used to calculate genetic distances.

Method	Genetic dissimilarity		
	Minimum	Maximum	Mean
Gower	0.067	0.501	0.254
Jaccard	0.000	0.989	0.321
Gower + Jaccard	0.067	1.430	0.620

The higher individual dissimilarities in the molecular data compared to quality data most likely accounted for the higher general mean in the former. As can be seen, the projections presented in Figure 2 were not error free since there was a small distortion in all cases. Since the grouping of genotypes into significant clusters was found to be impossible based on visual examination of the scatter plots, we undertook a cluster analysis based on Tocher's method (Tables 3 and 4). This analysis revealed 8 and 10 distinct groups for the 15 accessions based on quality and molecular data, respectively. Dots representing distinct groups of accessions were then differentiated in the scatter plot using different colours (Figure 2). Interestingly, nearly 34 % of 15 accessions occurred in a single group (Group B) based on quality and molecular information, thus confirming the previous conclusion regarding limited quality and molecular diversity in the tomato cultivated germplasm.

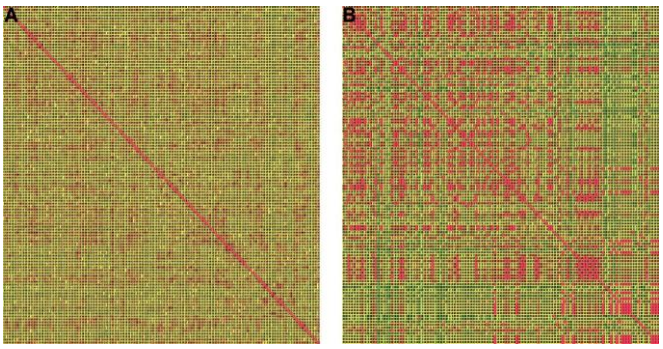

Fig 1: (A) Gower's dissimilarity matrix and (B) Jaccard's dissimilarity matrix. A colour gradient was used to graphically express the dissimilarity be-tween genotypes. Red indicates the most similar accessions, *i.e.*, lowest genetic dissimilarity, while green indicates the most dissimilar accessions, *i.e.*, lowest genetic similarity. A value of zero corresponded to the lowest genetic dissimilarity (represented by red) while 1 corresponded to the maximum genetic dissimilarity (represented by green). The dissimilarity matrices were symmetric and, consequently, the dissimilarity values below the diagonal were equivalent to those above the diagonal.

Table 3: Results for Tocher's cluster analysis based on Gower's dissimilarity matrix. Indistinguishable accessions (Gower's similarity = 1.0) were grouped in letter coded groups.

Group	No. of accession	Accession
A	1	Bhagya
B	5	Abhinav, GT-2, AT-3, AT-4, ToLCV RES-3
C	2	EC-589496, EC-520058
D	1	EC-500047
E	2	ATL-10-9, WIR-5032
F	1	IIHR-1966
G	1	IIHR-2101
H	2	DHT-14-8, ATL-10-7

Table 4: Results for Tocher's cluster analysis based on Jaccard's dissimilarity matrix. Accessions with indistinguishable multilocus marker genotypes (Jaccard's dissimilarity = 0) were grouped in letter coded groups.

Group	No. of accession	Accession
A	1	Bhagya
B	3	Abhinav, GT-2, AT-3
C	2	AT-4, ToLCV RES-3,
D	1	DHT-14-8
E	1	IIHR-1966
F	2	EC-589496, EC-520058
G	1	EC-500047
H	2	ATL-10-7, ATL-10-9
I	1	WIR-5032
J	1	IIHR-2101

Although the diversity analyses based on quality or molecular data resulted in a similar number of indistinguishable groups and a similar concentration of genotypes in a single group, the correlation between Gower's dissimilarity matrix and Jaccard's dissimilarity matrix was nevertheless low (Table 5). This finding indicates that molecular markers (IISRs and SSRs) are not sampling the same genomic regions that are relevant to quality differentiation of the accessions.

After these analyses, the quality dissimilarity matrix was combined with the molecular dissimilarity matrix generated using Jaccard's index. The combination was done by algebraically summing the matrices. No data standardization was required since these indices generated dissimilarity coefficients that varied between 0 and 1. The correlation between the joint dissimilarity matrix and the original dissimilarity matrices was then examined. As shown in Table 5, the new dissimilarity matrix displayed moderate correlation with the original matrices (quality or molecular).

This finding indicated a moderate degree of likelihood between this matrix and the original information, and that the procedure may be employed when one desires to use both quality and molecular information in a single analysis. The dissimilarity values in the joint dissimilarity matrix were used to measure quality + molecular diversity. This diversity varied from 0 to 1.43 among the 15 accessions, with an average dissimilarity among genotypes of 0.62 (Table 1). Figure 2 shows the combined dissimilarity matrix along with the 3D dispersion graph representing the relationship among genotypes (accessions) in terms of their joint qualitative and molecular diversity. Comparison of this graphical representation with the graphical representation of Gower's or Jaccard's dissimilarity matrices (Figure 1) suggests that the

joint dissimilarity matrix shares a high degree of likelihood with the original matrices (shown earlier by correlation analysis) and, at the same time, that this procedure is efficient in enhancing the sampling of genetic diversity. Such a matrix is therefore probably more realistic than the other matrices.

Table 5: Correlation coefficients between the dissimilarity matrices for quality and molecular data.

Matrices	Correlation
Jaccard (Jac) and Gower (Gow)	0.3750+*
Jaccard (Jac) and Joint (Jac + Gow)	0.99290++**
Gower (Gow) and Joint (Jac + Gow)	0.456626++**

*,** $p < 0.05$ and 0.01, respectively (*t*-test). +,++ $p < 0.05$ and $p < 0.01$, respectively (Mantel test with 1000 simulations). Gow: dissimilarity matrix obtained with Gower's method; Jac: dissimilarity matrix obtained with Jaccard's dissimilarity coefficient.

The results of Tocher's cluster analysis, based on the joint dissimilarity matrix, are presented in Table 6. Tocher's clustering method revealed an increase in the number of indistinguishable groups among the 15 accessions (8 groups). Interestingly, the largest group (group B) generated by the joint analysis of quality and molecular data contained only 34% of the 15 accessions. Compared to the largest group generated using quality and molecular independently, this number is nearly 1.50% smaller. Moreover, considering only this major group in the clustering generated by Tocher/Gower, Tocher/Jaccard and Tocher quality/molecular data analyses, 5 of the accessions that were grouped in Group B based on molecular markers were also grouped based on quality data, 5 of the accessions grouped in Group B based on quality/molecular data were also grouped based on quality data, only one accessions grouped in Group A, D, F and G based on quality/molecular data were also grouped based on molecular data and Three accessions were consistently grouped in D and E together, regardless of the dissimilarity matrix in which the cluster analysis was performed. This result indicates that some genotypes are always grouped together, regardless of the strategy used to study genetic diversity and should really be considered as duplicates or close genotypes when assessing germplasm conservation.

Table 6: Results of Tocher's cluster analysis based on a summed Gower and Jaccard dissimilarity matrix. Indistinguishable accessions were grouped in letter coded group profiles.

Group	No. of accession	Accessions
A	1	Bhagya
B	5	Abhinav, GT-2, AT-3, AT-4, ToLCV Res-3
C	1	DHT-14-8
D	3	EC-589496, EC-520058, EC-500047
E	3	ATL-10-7, ATL-10-9, WIR-5032
F	1	IIHR-1966
G	1	IIHR-2101

Together, these results indicate that the genetic diversity in the germplasm at disant hybridization farm is small, but not as limited as previously thought. These results also indicate that the joint analysis of quality and molecular data is effective in capturing all existing genetic variability.

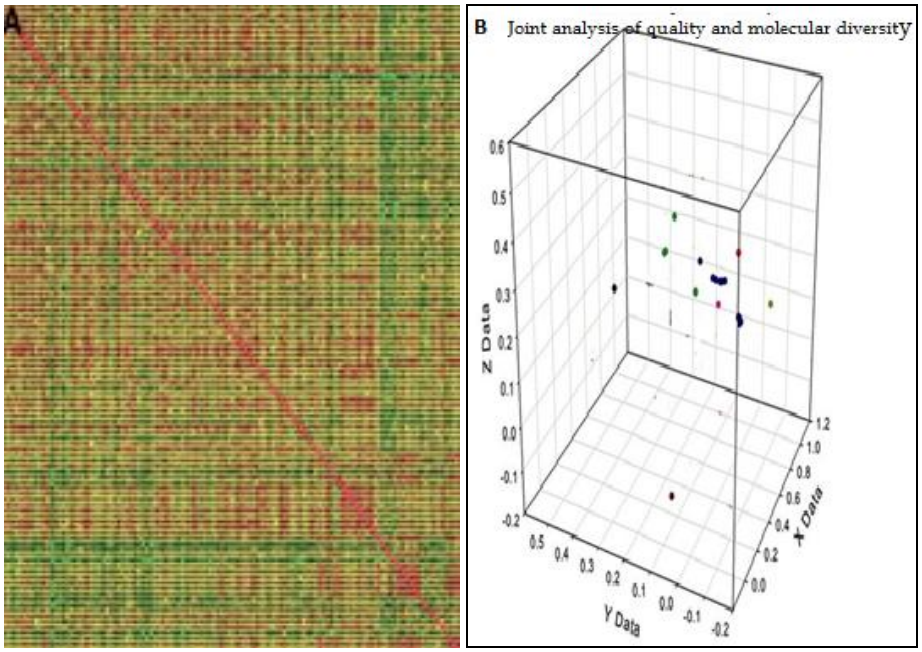

Fig 2(A): Joint dissimilarity matrix. A colour gradient was used to express the dissimilarity between genotypes. Red indicates the most similar accessions, *i.e.*, lowest genetic dissimilarity, while green indicates the most dissimilar accessions, *i.e.*, lowest genetic similarity. A value of zero corresponded to the lowest genetic dissimilarity (represented by red) while 1 corresponded to the maximum genetic dissimilarity (represented by green). The dissimilarity matrices were symmetric and, consequently, the dissimilarity values below the diagonal were equivalent to those above the diagonal. (B) 3D scatter plots showing the relationship among physic nut accessions assessed using phenotypic and molecular data jointly. The dots representing distinct groups of accessions (based on Tocher's cluster analysis) are show in different colours. X, Y and Z axis coordinates were calculated based on the distance projection. Note the different scales of the graph.

Discussion

To the best of our knowledge this is the most comprehensive assessment of the genetic diversity of the tomato germplasm in Distant Hybridization Farm, and one of the first to be based on quality and molecular data. The traits list encompassed quality and reproductive traits, including fruit yield, considered to be the most important trait to be improved in forthcoming years to make tomato a truly viable vegetable crop.

The extensive list of qualitative traits evaluated ensured that qualitative diversity data generated and, consequently, the results of this study, have a direct, fast application in the breeding program for more information regarding the genetic parameters and potential of this population for breeding purposes. The ISSR and SSR markers used in this study provided a robust platform for an initial assessment of tomato molecular diversity and allowed us to be confident about the conclusions reached for information regarding marker polymorphism, SSR allele frequencies, ISSR reproducibility, band in formativeness and primer resolving power.

Tomato genetic diversity was low based on both molecular and quality data. The average dissimilarity among the 15 genotypes was 0.32 and 0.19 based on molecular and quality data, respectively. These values agree with those previously reported for the most accessible tomato germplasm. (Zhou *et al.*, 2015; Bhattarai *et al.*, 2016 and Corrado *et al.*, 2014) [14, 1, 3]. According to Blanca *et al.* (2015) [2] the limited genetic diversity and considerable extent of duplications found in this germplasm collection despite its widespread origin and large size most likely reflects a common ancestry, drift and intensive selection of the currently cultivated material. Other recent reports that have sampled diversity using different molecular markers have reached similar conclusions regarding the limited variation in the most accessible germplasm resources of tomato in various countries (Tanksley, 2004) [12].

Comparison of the genetic dissimilarity determined here based on molecular markers with the data provided by Glogovac *et al.* (2013) [6] revealed a slight increase in the corresponding value. This may be related to the set of accessions used. Whereas Hassan *et al.* (2013) [7] analyzed 11 accessions our analysis was based on a set of 15 accessions, for which we had good quality quality data; A consequently, our optimized set tomato accessions consisted of the most diverse collection of tomato genotypes. This observation indicates that the germplasm collection can be optimized by maintaining only a set of the most diverse genotypes.

Together, these findings indicate that arbitrary molecular markers such as ISSR and anonymous markers such as SSR are not efficient in capturing the polymorphism that results in qualitative variation in tomato. This scenario could change if, instead of a small battery of ISSR markers and anonymous SSR, a battery encompassing thousands of single nucleotide polymorphisms (SNPs) densely covering the entire genome (and possibly every predicted gene-model) and EST-derived SSR are used. In this regard, some reports have indicated that when an adequate set of molecular markers is used to assess the genetic diversity of germplasm collections, the estimated molecular genetic distances can be successfully correlated with qualitative variation (Sim *et al.*, 2012) [11]. Since developed a large number of SNPs for tomato and in view of the availability of genomic resources (EST sequences generated using Illumina's GA IIx) that could be used to design primers to amplify EST-derived SSR (primer design is underway), a more detailed study of molecular diversity and of its relationship with qualitative variation is within the reach in the near future.

Our results indicate that tomato variability in distant hybridization farm is indeed limited (more than 50% of the average similarity), but not as much as previously thought. The slight change in the sampling intensity also affected the clustering of genotypes, and indicated that the joint analysis of quality and molecular data was effective in capturing most existing genetic variability. Considering Tocher's clustering based on the Gower and Jaccard dissimilarity matrix, respectively, joint analysis provided an increase of nearly 29% and 6% in the number of groups. Joint analysis also decreased the genotype concentration in a single group and was effective in discriminating among genotypes. Genotypes that were considered to be most diverse based solely on quality or molecular data were now grouped together with other genotypes. The opposite also occurred, *i.e.*, some genotypes that could not be correctly discriminated based solely on quality or molecular data were now placed in single accession groups.

Conclusion

These associations completely changed the picture regarding the genetic diversity of tomato in distant hybridization farm. However, considering that the tomato germplasm collection forms the basis of the breeding program for the species, it is important not only to identify the existing genetic diversity, but also to use the genetic diversity data to establish some guidelines. The finding that genetic dissimilarity among physic nut genotypes is indeed limited reinforces the need of adding more diversity to the germplasm bank to ensure long-term use of the germplasm in breeding programs. This initiative would be ideally performed by collecting new samples from the center of origin/diversity of the species in india and other countries.

Taken together, the results presented here demonstrate that: (1) the molecular diversity of the tomato does not resemble its qualitative variability, (2) the algebraic combination of Gower's qualitative dissimilarity matrix with Jaccard's molecular dissimilarity matrix can be used to successfully perform a joint genetic diversity analysis, (3) the combination of qualitative and molecular diversity matrices increases sampling of the tomato genetic diversity, (4) joint analysis of qualitative and molecular diversity reveals that tomato variability is indeed limited, but not as much as previously thought, and (5) additional collections are urgently needed to enhance the germplasm representativeness and usefulness in terms of species improvement.

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