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GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES, AND DNA-PROFILING IN PARTICULAR

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THE USE OF MOLECULAR MARKERS FOR THE LETTUCE SPECIES

Document prepared by experts from France

INTRODUCTION

1. The aim of this study is, in a first step, to evaluate the diversity of the *Lactuca sativa L.* species and to measure its evolution since 1952 (date of the opening of the French official catalogue of registration of varieties) to the present day. In a second step, it proposes a new tool based on the use of molecular markers for the management of reference collections in the DUS context with respect to the recommendations formulated within UPOV, as it has already been done in case of maize and barley species.

Background

2. Consumed for a very long time, the origin center of lettuce would be somewhere in Turkey and in the Caucasus or in the Middle East, where there is a great diversity of wild lettuce. Probably issued from a crossing between *Lactuca serriola L.* and another wild species (now extinct), lettuce (*Lactuca sativa L.*) is one of the most consumed fresh vegetables on all continents. Since its culture in ancient Egypt, then its introduction in Europe, the lettuce range is extended particularly with headed varieties.

3. Lettuce is a species with many morphological variations. Thus there are different cultigroups.

<table>
<tr><td colspan="7">LETTUCE – Lactuca sativa L.</td></tr>
<tr><td colspan="5">Lettuce which could form a head</td><td colspan="2">Not headed lettuce</td></tr>
<tr><td colspan="2">Oblong head and thick leaves</td><td colspan="3">Circular or flat head</td><td rowspan="2">Looseleaf</td><td rowspan="2">Chinese lettuce</td></tr>
<tr><td rowspan="2">Romaines
(also called Cos)</td><td rowspan="2">Latines</td><td rowspan="2">Butterhead</td><td colspan="2">Batavian</td></tr>
<tr><td>Summer crisp</td><td>Crisphead
(also called Iceberg)</td></tr>
</table>

Assessment of genetic diversity and species structure

4. In a competitive context of selection on a fairly uniform market like in Europe, new varieties are developed from a same genetic heritage considered as leader at the moment M.

5. The number of registered lettuce varieties has steadily increased since 1955. If we can well measure the number of registered varieties and its acceleration, what could we say about the actually present diversity relative to prior periods?

6. In this part, we study the allelic diversity :
   - present in each cultigroup,
   - existing at the moment M (to do that, dates of registration and withdrawal were taken into account, to reflect the commercial life of each variety),
   - introduced per period.

Materials and methods

7. Molecular data were provided on 500 varieties registered on the common catalogue of varieties of vegetable species (this number corresponds to 27% of the effective collection present at GEVES). This sub-set is representative of the 6 cultigroups present on the European sector (Romaine, Latines, Butterhead, Summer crisp, Crisphead and Looseleaf) and of 4 periods defined as follow : <1970, 1970-1985, 1985-2000 and >2000.

8. The 30 SSR used in this study were issued for 10 of them from Ivan Simko's publication in the *Journal of Heredity* in 2009. The 12 following were part of a study on the lettuce at the Netherlands (Van de Wiel *& al*, 1999). The last ones were developed by a private breeder.

9. After crushing the seeds with the Retsch MM301 crusher, DNA is extracted for a PCR. The analysis of the PCR results is done with GeneMapper software. The data are processed with PowerMarker V3.0 software. The distance genetic estimator chosen is the Rogers' distance (1972).

10. For each cultigroup and each period, a diversity index was calculated according to Nei's unbiased genetic diversity system (Nei 1978). The number of alleles and the allelic richness were also calculated. In order to appreciate the introduced diversity, only the varieties registered in a given period were taken into account. This allows to examine the repartition of the introduced alleles per period and to take stock of this diversity by comparing the diversity introduced :
   - in a first step, during the second period to one introduced during the first period,

- in a second step, during the third period versus the diversity introduced during periods1 and 2

- in a third step, during the fourth and last period of our study versus the diversity introduced during the 3 first periods.

11. Trees of representations of lettuce varieties have been made using DARwin software developed by CIRAD (Centre de coopération internationale en recherche agronomique pour le développement). Distances between varieties were represented graphically by Neighbor-Joining (Seitou and Nei, 1987). Bootstraps were made to control the robustness of the groups obtained. A visualization showing the diversification growing from root has been chosen.

Summary of Results

12. On the basis of the 500 lettuce varieties, 184 alleles have been put in evidence by the 30 SSR markers with between 4 to 17 alleles per locus.

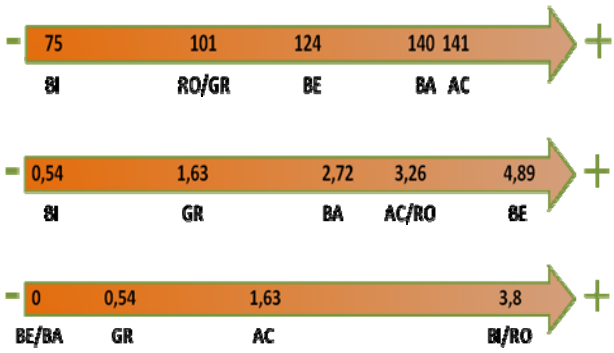
13. In a first time, the allelic diversity present in each cultigroup has been examined.

Allelic diversity per cultigroup

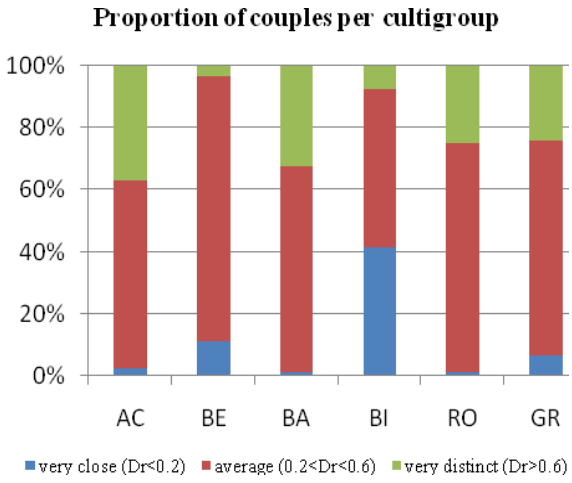
1) Regarding each cultigroup, the number of alleles put in evidence can pass from the simple to almost double.

2) A different proportion of alleles subservient to one cultigroup (that means allele only observed in one cultigroup) has been highlighted.

3) The proportion of alleles which are absent from a cultigroup is quite different.



AC : Loosleaf ; BA : Summer crisp ; BE : Butterhead ;
BI : Crisphead ; GR : Latines ; RO : Romaines



AC : Loosleaf ; BA : Summer crisp ; BE : Butterhead ;
BI : Crisphead ; GR : Latines ; RO : Romaines

14. Concerning genetic distances between varieties, the Crisphead cultigroup has the highest rate of very close varieties pairs (Dr<0.2) with 41,66%. Then come the Butterhead with 11,14 %, Latines with 6,67%, Loosleaf with 2,41%, Summer crisp with 1,21% and Romaines with 1,13%.

More than 1/3 of pairs of Loosleaf and Summer Crisphead varieties have a Rogers' distance greater than 0,6. For the Romaines and Latines types this rate goes down to ¼ while Crisphead and Butterhead have respectively 7,33% and 3,25% of pairs with a Rogers' distance greater than 0,6.

15. The average diversity index calculated per cultigroup is 0,54 for the Summer crisp type which constitutes the cultigroup with the highest index and represent 67,5 % of the theoretical maximal diversity. The next cultigroup is the Loosleaf type with an index of 0,52, then the Romaine type (0,49), the Latines (0,43), the Butterhead (0,37). The Crisphead cultigroup has the lowest index : 0,26 which represent 32,5% of the theoretical maximal diversity.

16. After this first overview of the diversity, the diversity existing in each period has been studied.

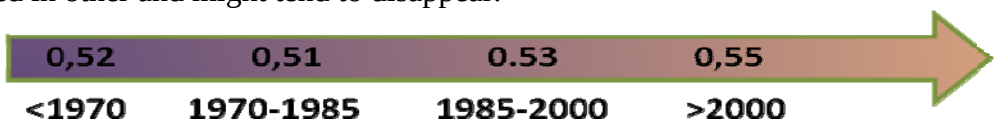
Allelic diversity existing at the moment M

	<1970	1970-1985	1985-2000	>2000
Number of varieties	**28**	**48**	**244**	**497**
Number of alleles put in evidence in the period	**116**	**129**	**166**	**184**
Allelic richness	**3.90**	**4.30**	**5.53**	**6.13**
% of varieties pair with Dr*<0.2	**0.26**	**2.04**	**3.19**	**1.93**
% of varieties pair with Dr*>0.6	**39.68**	**39.72**	**41.10**	**40.38**

**Dr = Rogers distance*

17. On the 4 period, the number of allele increases over time and in the same way the allelic richness. The proportion of very close varieties pairs (Dr<0,2) increases to 2000 (from 0,26% to 3,19%) for coming down to 1,93 %. The proportion of very distinct varieties pairs (Dr>0,6) remains stable over time in the approximately of 40%.

18. We also notice that the rare allele frequency continues to increase. These rare alleles can match new alleles which are introduced in small proportion, but also to old alleles that are being diluted in other and might tend to disappear.



19. In our sub-set, the Nei diversity index calculated per period tend to increase slightly over time (from 0,52 to 0,55). On the last period, this rate represents 68,75% of the theoretical maximal diversity.

20. In a third step, the allelic diversity introduced during each period has been scrutinized.

Allelic diversity introduced per period

	<1970	1970-1985	1985-2000	>2000
Number of new varieties	**28**	**20**	**199**	**253**
Number of alleles put in evidence in the period	**117**	**94**	**152**	**155**
Allelic richness	**3.90**	**3.13**	**5.07**	**5.17**
% of varieties pair with Dr*<0.2	**0.20**	**8.95**	**3.61**	**1.82**
% of varieties pair with Dr*>0.6	**39.68**	**38.95**	**40.81**	**36.73**

Dr = Rogers distance*

21. The number of alleles put in evidence during the second period (1970-1985) is the smallest. In addition, at the same period we notice the highest proportion of very close varieties pairs (Dr<0.2)

22. We observe that some alleles present in one period are not present among the new varieties listed the next period. During the period 1970-1985, new varieties have 70% of the alleles present in the previous period (< 1970). In the period 1985-2000, the new varieties have 89% of the alleles present in the 2 previous periods. The varieties registered after 2000 have 83% of the alleles present in the 3 previous periods.

23. The introduction of new alleles is not homogeneous over time. In the period 1970-1985, only 12 new alleles have been introduced whereas in the next period (1985-2000) this number is multiplied by 3 (37 new alleles). This progression is not confirmed in the last period (>2000) but the number of new alleles introduced (17) remains superior to the one of the period 1970-1985.

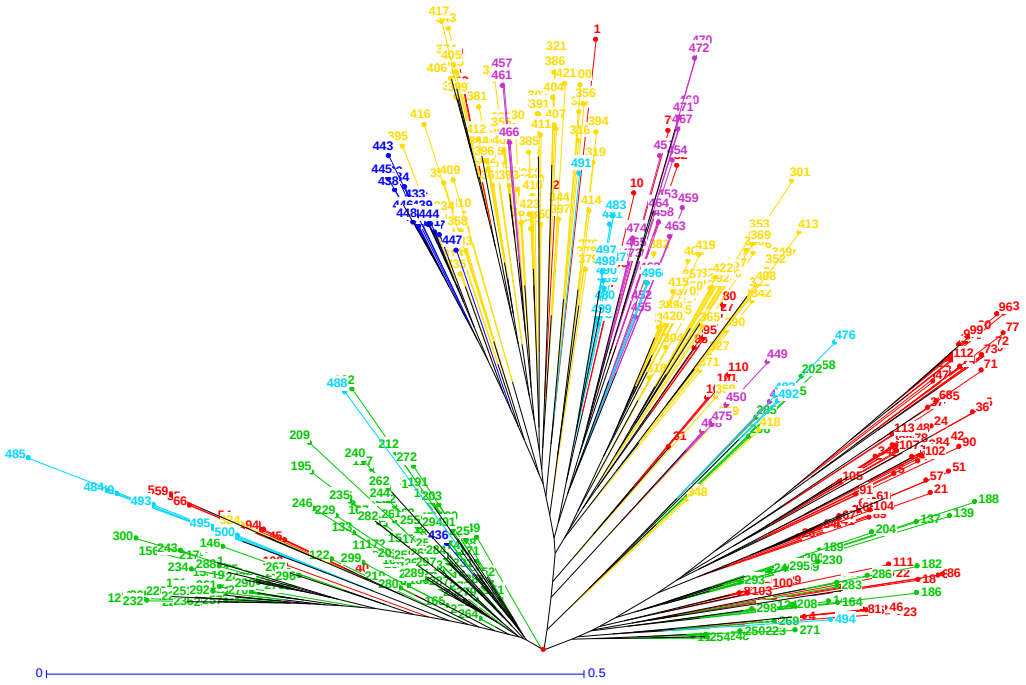
24. This introduction of new alleles is probably linked to the introgression of diseaseresistances often realized by interspecific crossing. Among the introduced alleles, we notice a proportion of rare alleles more increasingly important.

25. Nei diversity index calculated by period of registration confirms that the period 1970-1985 introduced slightly less diversity than others with an index equal to 0,48. Before this period, for the varieties registered before 1970, the index is 0,52. After 1985 and on the 2 periods studied (1985-2000 et >2000), the index stabilizes at 0,53.

26. Finally, this molecular study permits to realize dissimilarity matrix which allow to do some trees of representation of the 500 lettuce varieties of this subset.

Trees of representation of the 500 lettuce varieties

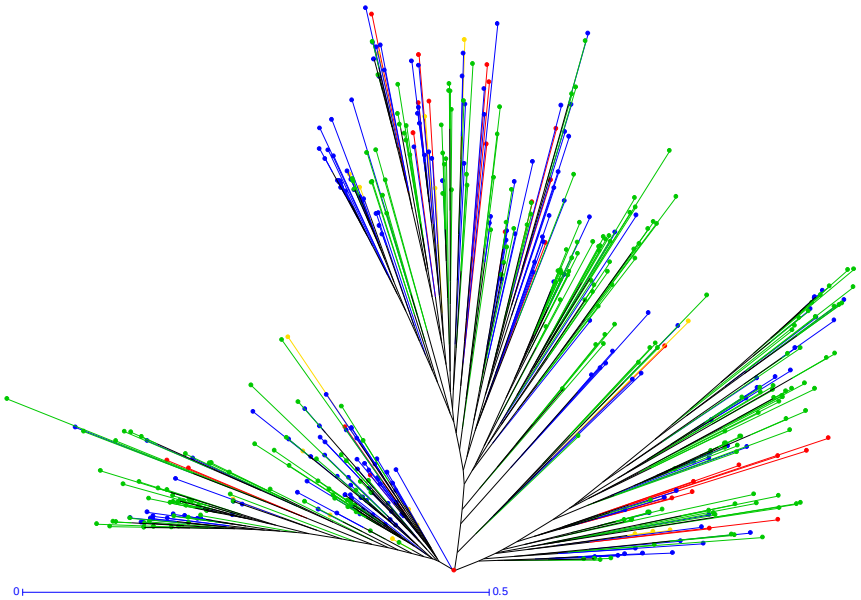
Visualization per cultigroup



Red : Looseleaf – Green : Butterhead - Yellow : Summer crisp Blue : Crisphead – Rose : Romaines – Turquoise : Latines

27. This visualization shows the structuring of this subset is based on the cultigroup. Although some varieties are placed in a cluster different from their cultigroup, we find large groups of varieties grown from a same cultigroup. A more thorough analysis also shows that this structuring can be observed according to the use of the variety (greenhouse or field).

Visualization per period of registration



Red : registration before1970 – Yellow : registration 1970 /1985 Bleu : registration 1985/2000 – Green : registration after 2000

28. If we look at our sample through the prism of the dates of registration, we observe that new varieties are never created from ancestral varieties but always from recent material

CONCLUSION

29. This study confirms a structuring of the lettuce species based on the cultigroups. Nevertheless, the observed diversity is unequalfrom one cultigroup to another and the distances between varieties are more or less close according to the studied cultigroup.

30. It also permits to identify periods which promote the introduction of a highest diversity (1985-2000 and >2000) and unlike a period forming a bottleneck of diversity (1970-1985). The introduction of new alleles is very linked with the introgression of disease resistances (to different races of downy mildew, lettuce mosaic virus, *Nasonovia ribisnigri*…) essentially done by interspecific crossing. We can make the hypothesis that the highest rate of rare alleles observed after 1985 has a link with new technologies of genome introgression which also allow the introgression of non-coding part. In the same time, an increase in mildew resistance determination, the emergence of *Nr* resistance but also the typological diversification, have been observed and highlighted in DUS trial.

31. We have seen a tendency to the increase in diversity. However, this diversity seems relative when we look at trees of representations of varieties. The proximity of varieties on the tree reflects their similarities from a genetic point of view.

32. We sometimes observe varieties issued from a cultigroup lost among varieties from another cultigroup. These varieties are the result of selection schemes which are more originals. For example, the variety 488, which is identified as a Latine type and is lost among Butterhead varieties, is the fruit of a crossing between a Butterhead variety and a Latine variety.

33. The second visualization per period shows that all new varieties are derived from an already existing genetic.

PROPOSITION OF A NEW TOOL FOR THE MANAGEMENT OF REFERENCE COLLECTION

34. Due to the increasing number of lettuce varieties of common knowledge, there is an increasing number of comparisons to be made between candidate varieties and varieties of common knowledge. It also complicates the choice of the control varieties and increases the size of the trial.

35. New sorting keys are needed. The question is : will the use of molecular data associated to phenotypic data to structure the lettuce reference collection be more efficient?

36. This approach has already been tested with success in maize and barley (see document BMT/DUS draft 6 “Possible Use of Biochemical and Molecular Markers in the Examination of Distinctness, Uniformity and Stability (DUS)”).

Materials and methods

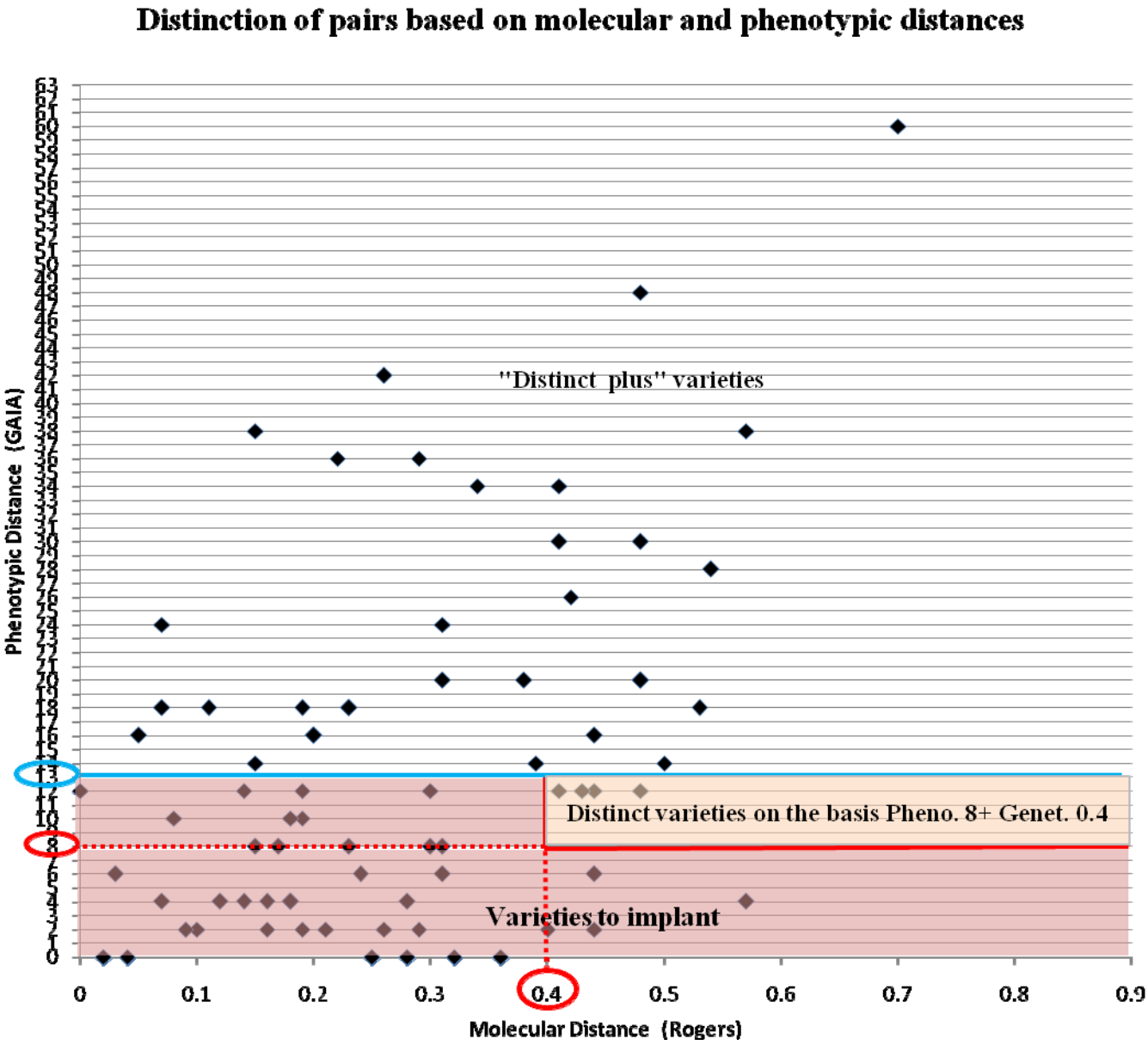
37. Crop experts were asked to observe different pairs of varieties grown side by side and to give a note using the following scale:

0 – the 2 varieties are similar or very close
1 – the 2 varieties are close but distinct
2 – the comparison is useful, but he varieties are clearly distinct
3 – the comparison could have been avoided, as the varieties are different
4 – the comparison should be avoided, as the varieties are very different

38. The obtained notes have been associated with the Rogers’ distances of each pair. This approach allows us to obtain 2 molecular thresholds: one for distinct varieties and one for “Distinct plus” varieties.

39. In order to calculate phenotypic distance, we also configure a lettuce application with the GAIA software (which has been developed by GEVES) based on matrix of 8 points to declare distinct varieties.

SUMMARY OF RESULTS



CONCLUSION

40. This molecular approach is still interesting. It proves itself in maize and barley species. In lettuce, the selected molecular threshold is 0.4. It is superior to the selected threshold in maize (0.2) which allows a 75% economy of varieties planted in the DUS trial and in barley (0.3) which allows a 50% economy of varieties planted.

41. At the moment, this combined approach does not significantly improve the actual tools. It does not surprise us so much as the lettuce is a self-pollinated and diploid species which has been worked since a very long time and its gene pool is not very extended. In fact, the actual tools are essentially based on (generally oligogenic) diseases resistances which allow us to reduce the pool of varieties to test. In species like maize or barley, this kind of tools doesn't exist. Therefore, the contribution of molecular data is larger than in lettuce.

42. However, in case of no new *Bl* appointment or in case of polygenic resistance (which could generate several phenotypes different from Resistant or Susceptible), this approach in lettuce could become very useful or even powerful.

GENERAL CONCLUSION

43. This study, on the basis of 500 lettuce varieties registered in the European Catalogue and 30 SSR markers, shows that there is a lettuce species structuring based on the cultigroup (and in some cases on the use –field or greenhouse) and that since 1985 the diversity increases over time. It is true that this diversity is uneven according to the observed cultigroup. It also confirms some expected: some cultigroup with a reduced genetic basis (Crisphead)) and few introgression of disease resistance gene shows little variability.

44. Despite this increase in diversity, varieties have relatively close genetic distances between them. Thus, the defined threshold of 0.4 does not allow to envisage the immediate construction a reference collection management tool on the basis of the SSR markers used here.

45. However, this molecular approach is very interesting and allows the characterization of 500 varieties on a little more than 1850 included in GEVES reference collection. A perspective would be to continue the genotyping of varieties to enable molecular characterization of these varieties. These data could then be useful to identify varieties for infringement procedures. They may also be used in procedures to investigate essential derivation.

(1) This study has been conducted by Stephanie CHRISTIEN in the framework of her engineer training in the GEVES unit of Brion (France) in cooperation with BioGEVES lab in Le Magneraud (France).

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