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Brassica selection criteria for the identification of the Most Appropriate Accessions (MAAs): relate to the **Brassica oleracea** *of Iberian Collection*

Final Report

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1. Introduction

The goal of AEGIS is "to create A European Genebank Integrated System for plant genetic resources for food and agriculture.

In selecting "European Accessions" choosing the Most Appropriate Accessions (MAAs) is an important step based on crop specific criteria. These selection criteria are meant to decide which accession of a group of duplicates to accept for inclusion in the European Collection.

For historical and geographical reasons the Iberian Peninsula is a center of genetic diversity for *Brassica oleracea*. It is a region with different linked centers of origin of biodiversity, allowing landraces to evolve adapted to the farming system.

The *B. oleracea* from Iberian Region is an important source of genetic diversity for Food and Agriculture. Portuguese Coles, "tronchuda" cabbage and "galega" kale are a rather unique, but diversified group of vegetables that can only be found in Portugal and Spain (Galicia) and in regions with a strong Iberian influence.

Considering the data analysis of the *B. oleracea* collections preserved *ex situ* in Europe, there is a significant B. *oleracea* collection and a large B. *oleracea* landrace collection conserved in the Iberian region (table 1, see Appendix 2).

The two institutions involved in the project (MBG, BPGV) represent the largest collections of *B. oleracea* landraces of *acephala*, *costata* and *capitata* from the Iberian Region.

- Banco Português de Germoplasma Vegetal and Misión Biológica de Galicia have *B. oleracea* landrace collections maintained in their country of origin, with a known origin and comprehensiveness of passport information.
- The Portuguese Brassica collection is conserved in the Banco Português de Germoplasma Vegetal (BPGV, INRB, I.P.) and the passport information of the collection is included in the CCDB for this crop (Bas, 2009) and in EURISCO. Actually, the collection holds 935 accessions from three species: 291 accessions from *B. rapa*, 102 from *B. napus* and **542 accessions from *B. oleracea***.. The national collection is a result of collecting missions that took place between 1990 and 2005. The accessions of the three species were collected in farms, and represent 88% of the total, being 84% originated from the North and Central part of Portugal.
- To preserve and characterize Galician landraces of the Brassica genus, a germplasm bank was created at the Misión Biológica de Galicia (MBG, CSIC) at Pontevedra, Spain. This gene bank keeps a Brassica collection of local landraces adapted to Atlantic conditions, which have been collected mostly from north-western Spain since the 1980s until the present. The passport data are included in EURISCO. Currently, the collection comprises 507 accessions from three species (*B. oleracea*, *B. rapa* and *B. napus*), including **250 accessions of *B. oleracea***.

To select the Most Appropriate Accessions (MAAs), it was considered the Iberian Collection composed of the Iberian accessions which are maintained in their respective countries of origin, at Banco Português de Germoplasma Vegetal (BPGV) and Misión Biológica de Galicia (MBG).

As part of effective management of these collections, probable duplicate accessions are routinely identified by the curators, both through the examination of passport data and/or field characterization. A part of the Iberian Collection was characterized using morpho-agronomic, molecular, and biochemical (glucosinolates, phenolic compounds and minerals) approaches. However, many aspects of the phylogeny within the species or cultivar groups and the classification of the different morphotypes need to be improved. In order to have a better understanding of the genetic variability of the Iberian landraces, to improve the classification of crop types and the rationalization of germplasm collections by reducing the number of duplicates, molecular markers could be useful. These goals are an important step for the selection of MAAS for the European Brassica collection.

The objectives of the project were:
i) apply the MAAs' selection criteria proposed for *B. rapa* to the Iberian *B. oleracea* collection, maintained in their respective countries of origin, at BPGV and MBG;
ii) apply molecular markers (ITS and SSR) to understand the genetic variability of the Iberian landraces, to improve the crop types classification and to eliminate duplicates;
iii) propose MAAs from the Iberian Collection.

To carry out the objectives, the activities scheduled were: **A1**: MAAs' selection criteria, **A2**: Germination monitoring, **A3**: Quantity of sample available, **A4**: Phylogenetic studies, **A5**: Evaluate the genetic variability of the Iberian landraces, **A6**: Meetings, **A7**: Scientific documentation. The project started on June of 2011 (see table 2, table 3, Appendix 2).

2. Materials and Methods

2.1 Accessions considered

Portuguese landraces (BPGV collection): [542 accessions of *Brassica oleracea* L. (6 capitata; 107 costata; 395 acephala)] and,
Spanish landraces (MBG collection): [250 accessions of *Brassica oleracea* L. (210 *acephala*; 2 *costata*; 38 *capitata*)].

2.2 Methods

The activities carried out by the different partners were as detailed in Appendix 2 (table 3)

Activity A1: **Apply the MAA selection criteria proposed for *B. rapa*** to the Iberian *B. oleracea* collection, maintained in their respective countries of origin, at BPGV and MBG.
The application of the MAA selection criteria was the responsibility of Centre for Genetic Resources (CGN) and Genetic Resources Unit of University of Warwick.
The method used was the examination of EURISCO descriptors and an investigation of whether the MAA selection criteria proposed for *B. rapa* could be applied to the *B. oleracea* Iberian collection. In the first meeting (activity A6) it was concluded that the MAA selection criteria proposed for *B. rapa* cannot be applied easily to the BPGV and MBG collections.
An alternative methodology selection was defined in first meeting: a preliminary dataset was proposed by BPGV and MBG and the accessions were selected according to collection site location.

Activities A2 (Germination monitoring) and **A3** (Quantity of sample available) were a responsibility of BPGV and MBG and the methodology followed for germination was the ISTA guidelines and the recommendations of AEGIS in its Quality Management System.
The non-germinating accessions were excluded. Accessions from Portugal are an original collection, not regenerated. Portuguese accessions from the 248 accessions were included when either the seed minimum quantity was 11 grams per accession and the germination percentage was above 50% or when the seed minimum quantity was above 100 grams per accession and the germination percentage was 40%.
Accessions from Spain were regenerated over the last years. Therefore, all accessions had a good germination percentage and enough seed quantity.

Activity A4: Phylogenetic studies
ITS chromosome region sequencing was applied to a specific group of accessions (92 accessions) resulting from activity A1 and A2 activities. The DNA extraction and ITS methodologies were defined in the 1st meeting (activity A6) and it was a responsibility of BPGV and the University of Porto. Two plants of each of the 92 *B. oleracea* germinate accessions were tested and additional sequences from Genbank (NCBI) were included.

a. *Biological material*

The DNA extractions were performed by BPGV on 57 *B. oleracea* accessions (26/31 *acephala*/*costata* accessions) with DNeasy Plant Mini Kit from QUIAGEN. The 70 DNA samples from MBG were obtained from two plants each of the 35 MAAs *B. oleracea* germinate accessions (19/16 *acephala*/*capitata* accessions), applying Liu and Whittier (1994) protocol with minor modifications. A total of 184 DNA samples were used for the ITS studies (Tab. 5, 6, see Appendix 2).

b. *ITS methodology*

Before amplification with ITS1 and ITS4, electrophoresis was performed to verify the DNA quality. The ITS1 and ITS4 regions were amplified by polymerase chain reaction (PCR), using standard primers (White et al., 1990). Amplifications were performed in 20 µL reactions, consisting of approximately 10 ng DNA template, 1 µM of each primer, 200 µM of each dNTP, 0.5U DyNazyme II DNA polymerase, 2 µL of 10X PCR buffer and 1.5 mM MgCl2. The amplification protocol consisted of an initial denaturation at 95°C for 2 minutes followed by 30 cycles of 95°C for 30s, 53°C for 30s and 72°C for 1 minute. A final extension step at 72°C for 7 minutes was completed. To estimate amplification efficiency, 5 µL of the PCR reaction product was electrophoresed using low-melting point 2% agarose gel in a 0.5x TBE buffer. PCR products were purified using the JetQuick (Genomed, Löhne, Germany) micro spin kit. Amplifications were sent for sequencing to Stab Vida (Madan Parque, Rua dos Inventores, s/n, sala 2.21, 2825-182 Caparica, Portugal).

The phylogenetic analysis using ITS 1 and ITS 4 was performed on 131 out of initial 184 DNA samples; 53 DNA samples were not able to be sequenced due to poor quality of DNA extracts and other reasons (Table 4, see Appendix 2). Between those 53 samples, there were 10 sequences from 5 accessions of different species, in database not identified correctly, and the ITS analysis permitted to verify. These sequences did not allow the alignment.
One hundred thirty one sequences were aligned with 41 available sequences from GenBank (NCBI, National Center for Biotechnology Information) for Brassica, using Clustal W with default conditions in the program BioEdit v5.0.9 (Hall, 1999). The sequence data was analyzed by neighbour-joining by using MEGA. Parsimony analysis and various clades were determined by MEGA (MEGA5 – Molecular Evolutionary Genetics Analysis http://www.megasoftware.net/).

Activity A5: Evaluate the genetic variability of the Iberian landraces

The DNA extraction and SSRs methodologies were defined during the 1st meeting (activity A6). A genetic diversity study by SSRs was a responsibility of MBG for the group of accessions resulted from A1 and A2 activities.

a. *Biological material*

DNA extractions were performed on 90 accessions using the same protocols as described for activity A4 (Tab. 5, 6, see Appendix 2). The differences were: BPGV made two bulks of 10 plants for each of the 55 accessions (24 *acephala* and *31costata*) from 20 seeds sown. MBG obtained DNA extracts from 20 individuals for each of 35 accessions (19/16 *acephala*/*capitata* crop types): the individual plants were sown in seedbeds and, 40 days later, the fourth or fifth leaf of each plant was used to perform DNA extractions following the method of Liu and Whittier (1994). Afterwards, DNA was mixed to produce two bulk sets per population formed by 10 individual samples each. For all DNA samples, the concentration of DNA was assessed spectrophotometrically (Spectra MR, DYNEX Technologies) and adjusted to 50ng/µl.

b. *SSRs methodology*

Ten SSRs were chosen from public data based on their polymorphism (Table 5, see Appendix 2) to carry out the analysis. Amplifications of SSRs were performed by using a PTC-100TM Thermal Cycler (MJ Research, Watertown, MA). The amplification consisted of a denaturing step at 95 ºC during 5 minutes followed by 35 denaturing cycles at 95 ºC for 30 sec, annealing at 56 ºC for 30 sec, and elongation at 72 ºC for 30 sec. The program ended with an extra elongation period of 10 minutes followed by a continuous cycle at 4 ºC. PCR reactions were carried out in a volume of 10µl containing 50 ng of each primer, 0.6 unit of Taq polymerase (BioTaq, Kapa Biosystems), 200 µM each dNTP, 1 × reaction buffer, 2.0 mM MgCl2, 50 ng DNA template, and distilled and autoclaved water. After amplification, SSR products were separated by electrophoresis in a capillary electrophoresis system (CEQ 8000, Beckman, Coulter).

Statistical analysis

For A5 activity, the statistical analysis was performed for 70 accessions out of initial 90 accessions, outcome of the activities A1, A2 and A4. Accessions with one of sequences in ITS studies were included in SSRs analysis.

Alleles were scored as present (1) or absent (0) for SSRs across accessions. The average number of alleles across loci was computed for each population. A similarity matrix with the presence-absence data was constructed by the NTSYS-PC version 2.1 (Rohlf 1998) based on the Dice coefficient, also known as the similarity coefficient of Nei and Li (1979). A band was considered as present in an accession if at least amplified in one bulk of the accession. The Dice coefficient is computed as 2a/(2a+b+c), where a is the number of SSR bands shared by genotypes in each pairwise comparison and b and c are the number of SSR bands present in each of the two genotypes and not present in the other. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) with the program NTSYS (Rohlf 1988). A co-phenetic correlation was calculated to test for the goodness-of-fit between similarity matrix obtained from the cluster and the original similarity matrix.

An analysis of molecular variance (AMOVA, Excoffier et al. 1992) based on the dissimilarity matrix of pairwise individuals was performed using Arlequin version 3.2 (Excoffier et al. 2005) to assess the genetic structure of the *B. oleracea* germplasm. This analysis partitioned the total SSR variation into that found within and among groups, that is among the tree crops: *capitata*, *acephala* and *costata*.. The significance level for variance components was tested by permutation test.

Activity A6: Two technical meetings with all project partners were held, one at BPGV (Portugal) on the 21st June 2011 and the other at MBG (Spain) on the 29th May 2012. The agendas of the meetings are included in the Appendix. During the second meeting, the Portuguese and Spanish colleagues felt they needed to meet once more,

to discuss the final analyses. An informal meeting between BPGV and MBG teams took place on 28th June, to discuss final molecular markers results.

3. Results

3.1 Apply the MAA selection criteria proposed for *B. rapa* to the Iberian *B. oleracea* collection, maintained in their respective countries of origin, at BPGV and MBG.

During the first meeting it was observed that:

i. The Iberian Collection represented in MBG and BPGV collections [1] conserves mainly landraces from the Northern Region of the Iberian Peninsula territory;
ii. Commercial varieties are less used and the landraces continue to be cultivated on farm;
iii. The accession names are local names and they are linked to the locality of origin.
iv. The same local names are repeated in different locations and different local names have been identified for the same landrace.

It was concluded that the MAA selection criteria proposed for *B. rapa* cannot be applied easily to the Iberian *B. oleracea* Collection, because the accession name (ACCENAME) is not a clear and discriminating descriptor, with synonymies between locations and landrace crop types.

During the first meeting (21st June 2011) the project partners have decided:

- To propose a new workflow (see Appendix 1) adapted to the Iberian Collection maintained at BPGV and MBG with MAA selection criteria for *B. oleracea* landraces.
- A subset of MGB and BPGV *B. oleracea* landrace collections was required. For the molecular analysis considering the project budget: accessions from the Northern Region of the Iberian Peninsula territory.
- Crop types *acephala, costata* and *capitata* were the most important in both countries.
- MGB has characterisation/evaluation data for some accessions – this information would be considered for the selection.
- The technical and financial capacity for the molecular work, within this project, was only allowing for the analysis of 100 accessions.
- The focus should be on the accessions from Entre Douro e Minho (Portugal) and Galicia (Spain) – westwards of around 8º 00' 00" W longitude.
- 50 acephala and a total of 50 *costata* and *capitata* accessions should be selected from the total dataset.

[1] Like it was explained in introduction concerning collections BPGV and MBG

A set of MGB and BPGV *B. oleracea* landrace collections was defined considering the first meeting decisions:

The preliminary dataset contained 248 accessions
MBG – 68 accessions: 50 accessions from *acephala* (kale) group and 18 accessions from *capitata* (cabbage) group; the accessions of *acephala* group proposed to constitute the future collection of Iberian MAAs were based on results, as stated in the paper of Padilla et al., 2007.
BPGV – 180 accessions, 32 from the *costata* group and 148 from the *acephala* group.

The dataset (248 accessions) was split by crop type and the accessions were selected based on:
√ Collection co-ordinates (geographic distribution)
√ Collection site elevation (eco-geographic variation)
√ Accession names were considered less important.

The accessions were selected according to geographical coordinates. *Acephala* and *costata* accessions were chosen based on their geographical location (longitude, latitude, and altitude) accessions. The collection location data were utilized to avoid selecting duplicates; the crop types are outcrossing and thus, the MAAs are assumed to be unique. The same was done for the *capitata* accessions except for two accessions from Betanzos (North of Galicia) because the region is an important area of *capitata* landraces and the accessions were collected at different dates.

Outcome

- The workflow of the MAA selection criteria for *B. oleracea* landraces was adapted and this permitted us to propose the MAAs for the *B. oleracea* dataset (see Appendix 1).

The *B. oleracea* Dataset selected of the Iberian Collection maintained at BPGV and MBG to apply the molecular markers (ITS and SSR) with the objectives to understand the genetic variability of the Iberian landraces, improve the classification of crop types and eliminate duplicates, is: 50 accessions of *acephala*, 18 accessions of *capitata* and 32 accessions of *costata* crop types (Fig. 1, see Appendix 3).

3.2 Germination monitoring and quantity of sample available

As a result of these two activities: 8 accessions need to be multiplied (< 100 grams) and 30 accessions with a lower germination rate (< 50%),need to be regenerated and require immediate attention, from the total of 248 accessions tested from the Portuguese collection.
From **100** accessions (64 BPGV/36 MBG accessions), outcome of A1 activity, 8 accessions did not germinate.

3.3 Phylogenetic analysis of 131 samples of *B. oleracea* accessions using ITS 1 and ITS4

The phylogenetic analysis using ITS 1 and ITS 4 was performed on 65 accessions (131 sequences) out of the initial 92 accessions (184 DNA samples) plus 41 available sequences selected from GenBank (NCBI, National Center for Biotechnology Information) of different species. The sequences from GenBank represent the genomic relationships between cultivated species of genus *Brassica* (see Table 4, Appendix 2, see Fig.2, Fig. 3, Appendix 3):

1. Clade 1 is composed of 70 sequences: 51 of *acephala* (33 from Portugal and 18 from Spain), 6 of *costata* from Portugal, and 13 of *capitata* from Spain.
2. Clade 2 is composed of 11 sequences: 5 of *costata* from Portugal and 6 of *acephala*, 5 from Portugal and 1 from Spain).
3. Clade 3 is composed of 41 sequences: 33 of *costata* from Portugal and 8 sequences of *acephala* (3 from Portugal and 5 from Spain).
4. Clade 4 is composed of 9 sequences from Portugal: 7 of *costata* and 2 of *acephala* (Table 4, Fig. 2, and Fig. 3, see Appendices 2, 3).
5. Some of accessions have one not two DNA samples sequenced.

The distribution of 41 sequences from GenBank was as follows:

a. Clade 1: 12 sequences of species *B. oleracea* capitata, *B. oleracea* botrytis, *B. napus*, *B. montana*.
b. Clade 2: 3 sequences of species *B. oleracea* acephala, *B. carinata*
c. Clade 3: 3 sequences of *B. oleracea*, *B. carinata*, *B. cretica* species
d. Clade 4: 23 sequences of *B. oleracea* botrytis*, B. napus*, *B. rapa*, *B. juncea*, *B. campestris* species (Fig. 3, see Appendix 3).

The samples, from the acephala group from Spain and Portugal were grouped in the same clades. The capitata samples were grouped with acephala group. The costata group was individualised in one clade (Fig. 2, see Appendix 3).

The study showed divergent results with a hypothesis that *costata* crop type is a hybrid between "galega" kale and cabbage (*capitata* crop type): the *costata* clade (clade 3) clustered with sequences of *B. oleracea*, *B. carinata*, *B. cretica* accessions from GenBank (NCBI). These results need. further study.

3.4 Genetic variability of the Iberian landraces

The genetic variability study was done for 8 *capitata*, 25 *costata* and 37 *acephala* accessions.

After screening 70 accessions with 10 microsatellites (SSRs), 67 alleles were found in the whole data set (Table 6, see Appendix 2). The average number of alleles by locus was 2.50. If accessions are classified by crop type, a total 63 alleles were found in *B. oleracea* var. *acephala* with an average of 2.6 alleles per locus; 40 alleles were found

in *B. oleracea* var. capitata with an average of 2.3 alleles per locus and 49 alleles were found in *B. oleracea* var. *costata* with an average of 2.5 alleles per locus.
Several Portuguese accessions had an average number of alleles per locus higher than 3.00, these are 01275-BPGV, 0176-BPGV, 01717-BPGV and 01653-BPGV (*B. oleracea* var. *acephala*) and 0287-BPGV and 0290-BPGV (*B. oleracea* var. *costata*).
Accessions with the lowest genetic diversity, below 2.00 were MBG-BRS0302, MBG-BRS0462, MBG-BRS0170 (*B. oleracea* var. *acephala*), 06283-BPGV, 06674-BPGV and 07800-BPGV (*B. oleracea* var. *costata*).

Cluster analysis showed seven ten groups for a genetic distance of 0.30 (Fig. 4, see Appendix 3).
Group **1** is composed by three Galician populations: two cabbages (MBG-BRS0400 and MBG-BRS0402) and one kale (MBG-BRS0462). Group **2** comprises 50 populations of kale (27 from Portugal and Galicia), cabbage (3 from Galicia) and tronchuda cabbage (20 populations from Portugal). Group **3** is formed by a Portuguese tronchuda cabbage (01778-BPGV). Group **4** is composed by a cabbage population from Galicia (MBG-BRS0076). Group **5** comprises 4 tronchuda cabbages from Portugal and 4 kales from Galicia. Group **6** is composed by one kale and one cabbage from Galicia. One kale from Portugal (02737BGPV) is present in group **7**. One population of cabbage from Galicia is present in group **8** (MBG-BRS0404). Group **9** is composed by 1 population of kale from Portugal (01725-BPGV) and 1 population of kale from Galicia (MBG-BRS0555). Finally, group **10** is formed by a single population from Galicia (MBG-BRS0302).

Accessions have been clustered independently from their geographic origin or their belonging to a crop type. The cophenetic correlation between the similarity matrix based on Dice coefficient and the similarity matrix obtained by the cluster was 0.76, which is a moderate value, meaning that cluster analysis is not a very good representation of the structure of the collection. Analysis of variance showed that most part of the variability (95.3%) is at the within-population-level and the rest of variability (4.7%) is located at the among-crop-type-level (Table 7, see Appendix 2), indicating that differences among accessions are bigger than differences among crop types. This maybe the reason, why cluster analysis does not classify accessions depending on the crop type they belong to.

3.5 Conclusions

a. The molecular markers showed that the workflow of the MAA selection criteria for *B. oleracea* landraces adopted permitted us to propose the MAAs for the *B. oleracea* dataset maintained at BPGV and MBG: the accessions were individualised and clustered independently from geographic origin or local name;
b. The kale accessions (*acephala* crop type) were present in all clusters;
c. The *costata* and *capitata* crop types are distributed over different groups;

d. It was not observed that crop types clustered by location;
e. Two samples are very similar (06439BPGV is *acephala* accession, 01864BPGV is *costata* accession) (Figures 4, Appendix 3): they were collected in different year and in different local but the seed sample of *costata* accession has mix of crop types seeds (kale and *costata*),
f. The kale (*acephala*) accession 02170BPGV has mix with *costata* seeds and it is similar with the *costata* sample (01779BPGV);

In ITS tree those sequences were grouped in two different clades (Figures 3, Appendix 3).
For MAAs proposal were not selected the *costata* accession (01864BPGV) and kale accession (02170BPGV).

Were identified the duplicates?
The smallest genetic distance verified was between Portuguese accessions, especially between the different crop types ("galega" and "tronchuda"), followed by two *costata* ("tronchuda") clusters with two accessions, each one from a different location. The main reasons for the small genetic distances can be explained by the seed exchange between farmers from different locations (*costata* accessions from different location) or; the crop system ("galega" is biannual and some plants are left to seed harvest without isolation and more susceptibly to out crossing. Seed harvest of "tronchuda" is annual and with unconventional isolation: the isolation is spatial without cages, and the neighbouring farmers will grow the same crop types). One more reason to consider, and which occurred in this study, is the mixture of seeds of different crop types from the same species. The situation can occur for the reasons previously reported and associated with crop system, even more, in the case of landraces. The seeds of different crop types of *B. oleracea* species are similar. This element is an essential point in the collections management.

The results obtained from SSRs and ITS studies (Table 8, see Appendix 2, Figures 3, 4, Appendix 3) showed the same type of clustering:

- Large clusters and clades with accessions from different crop types and locations. Nevertheless, the *costata* crop type was phylogenetically classified by ITS markers as one distinct group;
- Even with a small number of loci studied, the genetic variability was verified;
- The ITS markers allowed identified the accessions not correctly identified and crossing with SSRs information it was possible identify the samples with mixture of seeds from different crop types of *B. oleracea* species;
- The ITS and SSRs markers can be tools for to select MAAs of landraces: allows the genetic variability representation, identify the duplicates, identify the accessions with mix of seeds and the incorrect identification of species.

Once the ITS and SSRs analyses realised, a set of 42 accessions, maintained at BPGV and MBG (18 from Portugal and 24 from Spain) was selected as MAAs. The accessions

selection presentation considered the variability between dendogram clusters and the germination-quantity indicators.

The project has shown that for these conditions, for each location, even the genetic base can be small, the farmers' selection resulted in morphologically distinct landraces, as can be seen in other studies (Dias and Monteiro, 1994).

The proposed criteria, for the selection of MAAs for the Iberian *B. oleracea* landraces from BPGV and MBG, can be used as a model for selection of MAAs of other Brassica landrace collections. The ITS and SSRs markers are methodologies to be used to test the MAAs proposal.

One scientific document ((1), see Appendix 5) was submitted to an International meeting (Brassica 2012): 6th International Symposium on Brassica and 18th Crucifer Genetics Workshop "Exploitation of Brassica diversity for improving agriculture chains" which will be held in November 2012.
This project resulted in more information produced that we are preparing to publish.

4. Final recommendations

A set of 42 candidate European Accessions will be proposed, from the Brassica Iberian Collection, maintained at BPGV and MBG (Table 9 see Appendix 2) to the respective National coordinators and flagged in EURISCO.

SSR and ITS methods proved to be valid, important and applicable to the Brassica landraces collections. Molecular data is a reliable instrument for the identification of European accessions of *Brassica olereacea*.

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Appendix 4 – Agenda meetings

Appendix **1**

Proposed Criteria for the selection of MAAs for *B. oleracea* landraces from Iberian Peninsula

a. Database management by EURISCO descriptors

Check the fields DONORNUMB, DONORCODE, SAMSTAT

to identity the accessions from other collections

exclude duplicates

Include accessions agronomically and/or historically/culturally important

b. Sort by different crop types (SUBTAXA)

c. Sort by different geographic origin (ORIGCITY) for landraces by each crop type

d. Check the field ACCENAME:

- Accession name are mostly local names
- The same local names (to identify accessions) have been used in different locations and different names were found for the same landrace

Step 1. Select accession with unique ACCENAME

Step 2. Accessions with the same ACCENAME and diffrent COLLSITE

2.1 **Select** accessions from diferent geographic distribution

Different location of collecting site (COLLSITE)

2.2 Accessions with the same ACCENAME and same COLLSITE

a. **Select** accessions from

1) Different co-ordinates [(LATITUDE) and (LONGITUDE) of collecting site]

2) Different site altitude [ALTITUDE of collecting site] and

3) Selected accessions using characterization database

b. Accessions from the same co-ordinates and site elevation

1) **Select** accessions collected in different years ((COLLDATA) Collecting data of sample) and using characterization database

Accessions without characterization database

2) **Select** accessions collected in different years and with one cycle of regeneration.

Step 3. Accessions with different ACCENAME from same co-ordinates and site elevation

Select accessions by characterization database and with one cycle of regeneration

Step 4. Accessions without ACCENAME

Select accession with unique COLLSITE, co-ordinates and site elevation by characterization database

Appendix **2**

Table 1. *Brassica oleracea* collections in: Europe, Iberian region, in Portuguese genebank (BPGV) and in Misión Biológica de Galicia genebank (MBG)

Number of accessions	**Conservation *ex situ* (European *B. oleracea* Database)**						BPGV collection	MBG collection
	Europe	**Iberian region**	**In Iberian Institutions**					
			SP	**SP origin**	**PRT**	**PRT origin**		
Total	11,737	3,603	1,281	1,119	573	573	542	250
Landraces	3,373	1,362	765	479	542	542	542	250

Table 2. Workplan implementation – Activities by project partners

Main activities	**Project Partners**				
	BPGV	UP	MBG	GRU	CGN
A1: MAAs criteria selection	X		X	X	X
A2: Germination monitoring	X		X		
A3: Quantity of sample available	X		X		
A4: Phylogenetic studies	X	X			
A5: Evaluate the genetic variability of the Iberian landraces	X		X		
A6: Meetings at BPGV	X	X	X	X	X
A7: Scientific doc elaboration and reports	X	X	X	X	X

Table 3. Workplan implementation – Timetable of Project Activities

Main activities	J	F	M	A	M	**J**	J	A	S	O	N	D
A1: MAAs criteria selection												
A2: Germination monitoring												
A3: Quantity of sample available												
A4: Phylogenetic studies												
A5: Evaluate the genetic variability of the Iberian landraces												
A6: Meetings												
A7: Scientific doc elaboration and reports												

Table 4. Identification of DNA samples of BPGV accessions to ITS and SSRs methodology (not germinate accessions / not sufficient plants to bulk)

Accession number	Costata group "tronchuda" **C**	**Accession number**	Kale group "Galega" **A**
01627-BPGV	1	**01275-BPGV**	33
01726-BPGV	2	**01653-BPGV**	34

01729-BPGV	3	**01706-BPGV**	35
01739-BPGV	4	**01717-BPGV**	36
01747-BPGV	5	**01725-BPGV**	37
01773-BPGV	6	**02018-BPGV**	38
01778-BPGV	7	**02029-BPGV**	39
01779-BPGV	8	**02165-BPGV**	40
01782-BPGV	9	**02170-BPGV**	41
01783-BPGV	10	**02580-BPGV**	42
01794-BPGV	11	**02595-BPGV**	43
01796-BPGV	12	**02737-BPGV**	44
01839-BPGV	13	**02946-BPGV**	45
01862-BPGV	14	**03006-BPGV**	46
01864-BPGV	15	**03091-BPGV**	47
02075-BPGV	16	**03544-BPGV**	48
02076-BPGV	17	**03699-BPGV**	49
02082-BPGV	18	**03713-BPGV**	50
02087-BPGV	19	**03744-BPGV**	51
02090-BPGV	20	**04308-BPGV**	52
02578-BPGV	21	**04693-BPGV**	53
02636-BPGV	22	**06338-BPGV**	54
06250-BPGV	23	**06392-BPGV**	55
06275-BPGV	24	**06439-BPGV**	56
06283-BPGV	25	**06459-BPGV**	57
06387-BPGV	26	**06567-BPGV**	58
06419-BPGV	27	**06583-BPGV**	59
06456-BPGV	28	**07243-BPGV**	60
06674-BPGV	29	**07795-BPGV**	61
06812-BPGV	30	**07799-BPGV**	62
07800-BPGV	31	**08804-BPGV**	63
08362-BPGV	32		

Table 5. Identification of DNA samples of MBG accessions to ITS and SSRs methodology

Accession number	MBG accessions **E**	Crop type
MBG-BRS0462	2	Kale
MBG-BRS0302	6	Kale
MBG-BRS0205	10	Kale
MBG-BRS0399	15	Kale
MBG-BRS0158	17	Kale
MBG-BRS0446	19	Kale
MBG-BRS0493	28	Kale
MBG-BRS0211	30	Kale
MBG-BRS0560	32	Kale
MBG-BRS0072	40	capitata
MBG-BRS0076	41	capitata
MBG-BRS0083	42	capitata
MBG-BRS0120	43	capitata

MBG-BRS0400	45	capitata
MBG-BRS0404	46	capitata
MBG-BRS0408	47	capitata
MBG-BRS0411	48	capitata
MBG-BRS0425	49	capitata
MBG-BRS0057	51	capitata
MBG-BRS0402	52	capitata
MBG-BRS0452	53	capitata
MBG-BRS0161	96	Kale
MBG-BRS0162	97	Kale
MBG-BRS0170	98	Kale
MBG-BRS0192	99	Kale
MBG-BRS0301	100	Kale
MBG-BRS0396	101	Kale
MBG-BRS0406	102	Kale
MBG-BRS0410	103	Kale
MBG-BRS0397	105	capitata
MBG-BRS0409	106	capitata
MBG-BRS0449	107	capitata
MBG-BRS0555	108	Kale
MBG-BRS0074	109	capitata
MBG-BRS0500	110	Kale
MBG-BRS0078	Seed did not germinate	capitata
MBG-BRS0176	Seed did not germinate	capitata

Table 6. Clade distribution of samples for the three groups of Brassica

	Group A	Group C	Group E[2]
Clade 1	33	6	31
Clade 2	5	5	1
Clade 3	3	33	5
Clade 4	2	7	0
Sub total1	**43**	**51**	**37**
Not germinated	10	2	4
Not sequenced	9	11	33
Sub total2	**19**	**13**	**37**
Total	62	64	74

[2] The group E is a mix from MBG collection with 13 samples of capitata accessions and 24 samples of acephala accessions. Groups A and C are from BPGV collection and the Group A is of acephala accessions and C group is of costata accessions.

Table 7. SSR markers employed to characterize 70 populations from BPGV and MBG

SSR	Reference	Forward primer	Reverse primer
Na14-E08	Lowe et al. (2003)	TTACTATCCCCTCTCCGCAC	GCGGATTATGATGACGCAG
Na12-E05	Lowe et al. (2003)	CGTATGTTTGTTCCACCTGC	ACTAGCAACCACAACGGACC
OI10-H02	Lowe et al. (2003)	AACAGGAAGAAACGACGAGG	AGAGAGCCATGAGAAGCACC
BRMS-037	Suwabe et al. (2004)	CTGCTCGCATTTTTTATCATAC	TACGCTTGGGAGAGAAAACTAT
Na10-H03	Lowe et al. (2003)	GAGCTGGCTCATTCAACTCC	CACAATTTCTCAGACAAAACGG
Na10-DO9	Lowe et al. (2003)	AAGAACGTCAAGATCCTCTGC	ACCACCACGGTAGTAGAGCG
FITO 036	Iñiguez-luy et al. (2008)	GGATTGCCTGAGTTTATTCTT	TCTGGAGTAGATGCTTTGGT
FITO 139	Iñiguez-luy et al. (2008)	CCTCCATTACCACCACAA	CGTAGACAAACAACACCTGA
Ol10-F11	Lowe et al. (2003)	TTTGGAACGTCCGTAGAAGG	CAGCTGACTTCGAAAGGTCC
Na12-A02	Lowe et al. (2003)	AGCCTTGTTGCTTTTCAACG	AGTGAATCGATGATCTCGCC

Table 8. Total number of alleles and average number of alleles by locus

Cultivar	Var.	No. Alleles	No. Alleles by locus
Acephala crop		63	2.60
Capitata crop		40	2.30
Costata crop		49	2.50
Total dataset		67	2.50

Table 9. Molecular analysis of variance (MANOVA)

Source of variation	Sum of squares	Variance components	Percentage of variation
Among crops	29.46	0.31	4.7
Within crops	529.98	6.25	95.3
Total	559.43	6.56	

Table 10. Classification of accessions by SSRs (cluster) and ITS (clades) markers dendograms (a bold were identified the PRT accessions selected to MAAs proposal; the MBG accessions were selected all)

Acc.	Var.	Cluster	Clades	LOCNAME	PROVINCE
01275-BPGV	acephala	2	1	Couve	Melgaço
01653-BPGV	acephala	2	1	Couve galega	Ponte de Lima
01706-BPGV	acephala	2	1,2	Couve galega	Ponte de Lima
01717-BPGV	acephala	2	2,3	Couve galega	Melgaço
01725-BPGV	acephala	9	1	Couve galega	Braga
02018-BPGV	acephala	2	2,3	Couve galega	Lousada

02029-BPGV	acephala	2	1	Couve galega frisada	Felgueiras
02170-BPGV	acephala	2	2,3	Couve galega	Monção
02737-BPGV	acephala	7	1	Couve galega	Caminha
02946-BPGV	acephala	2	1	Couve galega	Fafe
03006-BPGV	acephala	2	1	Couve galega	Guimarães
03699-BPGV	acephala	2	1,2	Couve galega	Cab. Basto
03713-BPGV	acephala	2	1	Couve galega	Cab. Basto
03744-BPGV	acephala	2	1	Couve galega	Cab. Basto
04308-BPGV	acephala	2	1	Couve	Monção
06338-BPGV	acephala	2	1	Couve galega	Terras Bouro
06439-BPGV	acephala	2	1	Couve galega	Terras Bouro
06583-BPGV	acephala	2	1	Couve galega	Vieira do Minho
07243-BPGV	acephala	2	1	Couve galega	Arcos Valdevez
07795-BPGV	acephala	2	1	Couve galega	Amares
07799-BPGV	acephala	2	1	Couve galega	Amares
MBG-BRS0161	acephala	5	1	Berza	Melón
MBG-BRS0162	acephala	5	1	Berza	A Cañiza
MBG-BRS0170	acephala	2	1	Berza	Ribadavia
MBG-BRS0205	acephala	2	1,3	Berza	Bande
MBG-BRS0211	acephala	5	1	Berza	Cerdedo
MBG-BRS0301	acephala	2	1	Berza	Muiños
MBG-BRS0302	acephala	10	1	Berza	Calvos de Randín
MBG-BRS0396	acephala	2	1	Coles?	Coirós
MBG-BRS0406	acephala	2	1	Berza	Vimianzo
MBG-BRS0410	acephala	2	1	Berza	Ponteceso
MBG-BRS0446	acephala	2	2	Berza lisa	Cotobade
MBG-BRS0462	acephala	1	1,3	Berza gallega morada	Melide
MBG-BRS0493	acephala	2	1	Coles	Ordes
MBG-BRS0500	acephala	5	3	Berzas	Mazaricos
MBG-BRS0555	acephala	9	1	Verdure blanca	Tui
MBG-BRS0560	acephala	6	3	Coles	Touro
MBG-BRS0057	capitata	2	1	Repollo	Bueu
MBG-BRS0072	capitata	6	1	Repollo	Poio
MBG-BRS0076	capitata	4	1	Repollo corazón de buey	Poio
MBG-BRS0083	capitata	2	1	Repollo	Forcarey
MBG-BRS0400	capitata	1	1	Repollo	Coirós
MBG-BRS0402	capitata	1	1	Repollo	Betanzos
MBG-BRS0404	capitata	8	1	Berza	Zas
MBG-BRS0452	capitata	2	1	Repollo	Narón
01627-BPGV	costata	2	2,3	Couve tronchuda	Ponte Lima
01726-BPGV	costata	2	1	Couve tronchuda	Braga
01739-BPGV	costata	2	3	Couve tronchuda	Amares
01747-BPGV	costata	2	3	Couve penca de Gondomar	Gondomar

01773-BPGV	costata	2	3	Couve penca asa de cântaro	Gondomar
01778-BPGV	costata	3	3	Couve tronchuda	Barcelos
01779-BPGV	costata	2	3	Coivão	Barcelos
01782-BPGV	costata	2	3	Coivão pé alto	Esposende
01783-BPGV	costata	2	3	Couve penca	Esposende
01794-BPGV	costata	2	3	Coivão	Esposende
01839-BPGV	costata	2	1	Coivão	Barcelos
01862-BPGV	costata	2	3	Couve tronchuda	Póvoa do Lanhoso
01864-BPGV	costata	2	3	Couve penca	Póvoa do Lanhoso
02075-BPGV	costata	2	2,3	Couve tronchuda	Póvoa do Lanhoso
02076-BPGV	costata	2	3	Couve tronchuda	Póvoa do Lanhoso
02082-BPGV	costata	2	3	Couve tronchuda	Póvoa do Lanhoso
02087-BPGV	costata	2	3	Couve tronchuda	Póvoa do Lanhoso
02090-BPGV	costata	2	1	Couve tronchuda	Póvoa do Lanhoso
06250-BPGV	costata	2	3	Couve tronchuda	Terras Bouro
06275-BPGV	costata	2	3	Couve tronchuda	Terras Bouro
06283-BPGV	costata	5	2,3	Couve tronchuda	Terras Bouro
06387-BPGV	costata	5	2,3	Couve tronchuda	Terras Bouro
06419-BPGV	costata	5	1,3	Couve tronchuda	Terras Bouro
06674-BPGV	costata	5	2	Couve tronchuda	Vieira Minho
07800-BPGV	costata	2	3	Couve tronchuda	Amares

Table 11. Final proposal of candidate European Accessions

ACCENUMB	Subtaxa	Country
01627-BPGV	costata	PRT
01706-BPGV	acephala	PRT
01717-BPGV	acephala	PRT
01725-BPGV	acephala	PRT
01747-BPGV	costata	PRT
01773-BPGV	costata	PRT
01782-BPGV	costata	PRT
01783-BPGV	costata	PRT
01794-BPGV	costata	PRT
01839-BPGV	costata	PRT
02018-BPGV	acephala	PRT
02029-BPGV	acephala	PRT
02087-BPGV	costata	PRT
02090-BPGV	costata	PRT
04308-BPGV	acephala	PRT
06439-BPGV	acephala	PRT
07795-BPGV	acephala	PRT

07799-BPGV	acephala	PRT
MBG-BRS0057	capitata	SP
MBG-BRS0072	capitata	SP
MBG-BRS0076	capitata	SP
MBG-BRS0083	capitata	SP
MBG-BRS0161	acephala	SP
MBG-BRS0162	acephala	SP
MBG-BRS0170	acephala	SP
MBG-BRS0205	acephala	SP
MBG-BRS0211	acephala	SP
MBG-BRS0301	acephala	SP
MBG-BRS0302	acephala	SP
MBG-BRS0396	acephala	SP
MBG-BRS0400	capitata	SP
MBG-BRS0402	capitata	SP
MBG-BRS0404	capitata	SP
MBG-BRS0406	acephala	SP
MBG-BRS0410	acephala	SP
MBG-BRS0446	acephala	SP
MBG-BRS0452	capitata	SP
MBG-BRS0462	acephala	SP
MBG-BRS0493	acephala	SP
MBG-BRS0500	acephala	SP
MBG-BRS0555	acephala	SP
MBG-BRS0560	acephala	SP

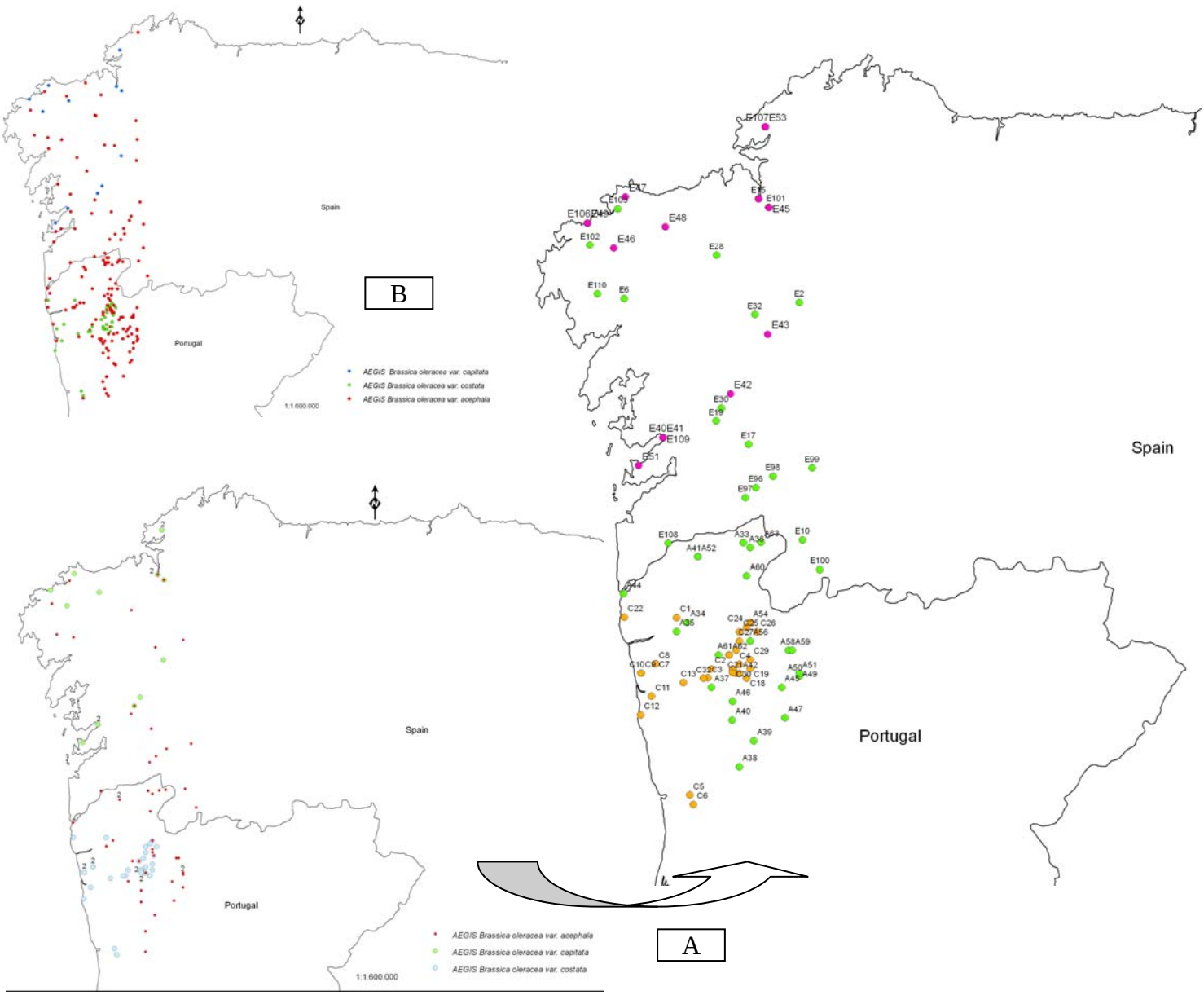


Fig. 1. Location of MAAs *B. oleracea* Dataset of Iberian collection (50 accessions of acephala, 18 accessions of capitata and 32 accessions of costata) (A) obtain by applying to a preliminary dataset (B) the selection criteria workflow adapted to Iberian collection of *B. oleracea* landraces.

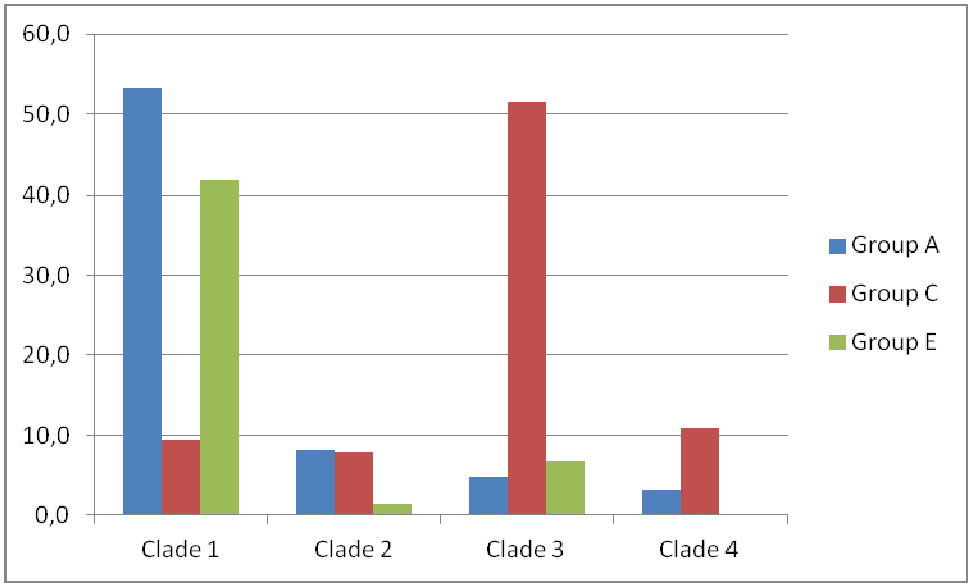


Fig. 2. Percentage of accessions by clade[3].

[3] The group E is a mix from MBG collection with 13 samples of capitata accessions (C) and 24 samples of acephala accessions (A). Groups A and C are from BPGV collection and the Group A is of acephala accessions and group C is of costata accessions.

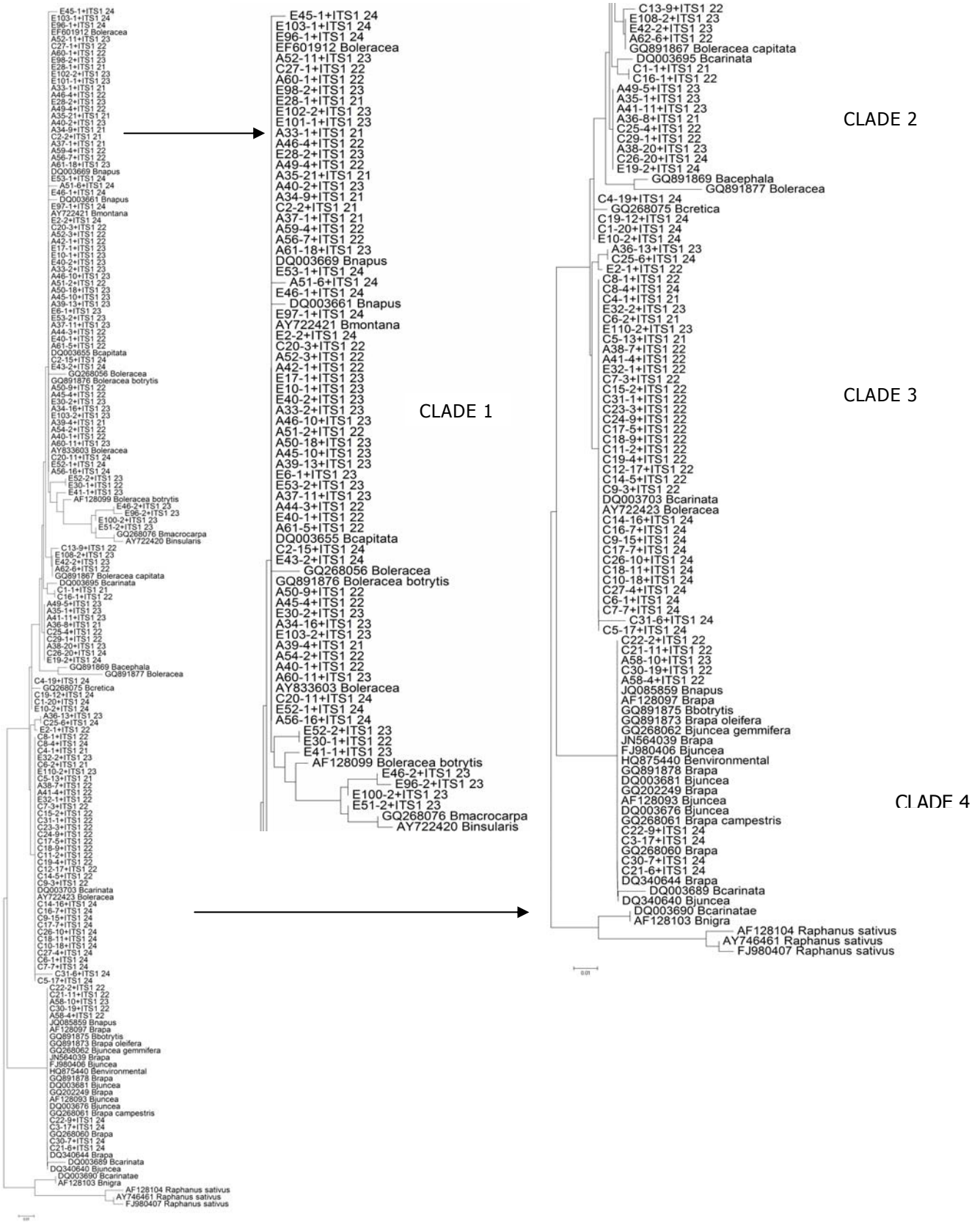


Fig. 3. *B. oleracea* tree obtain with 131 sequence data analyzed by neighbour-joining. Sequences composition of clades: codes A/C to acephala/costata DNA samples of Portuguese accessions; code E a mix from Spain collection with 13 samples of capitata accessions and 24 samples of acephala accessions

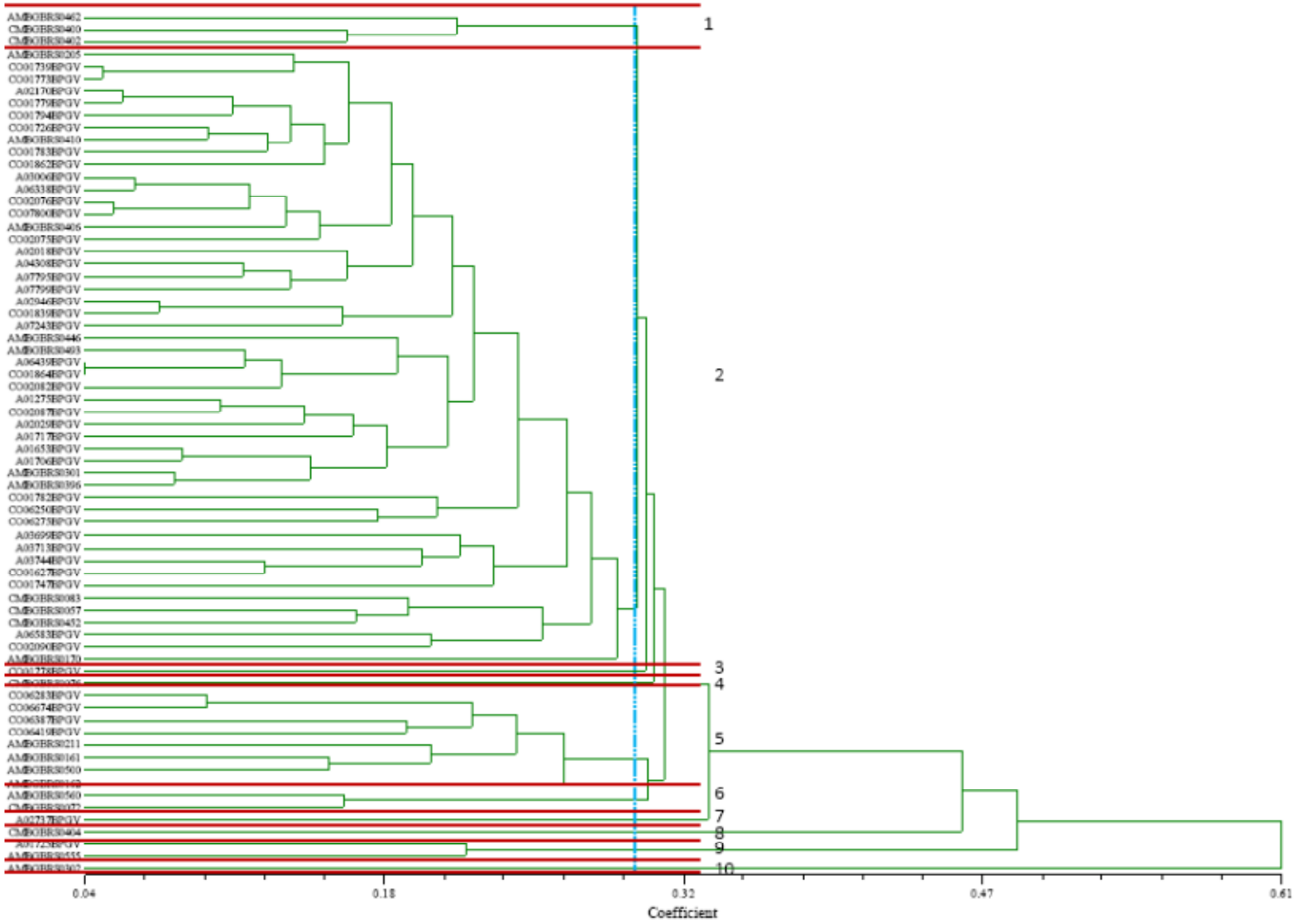
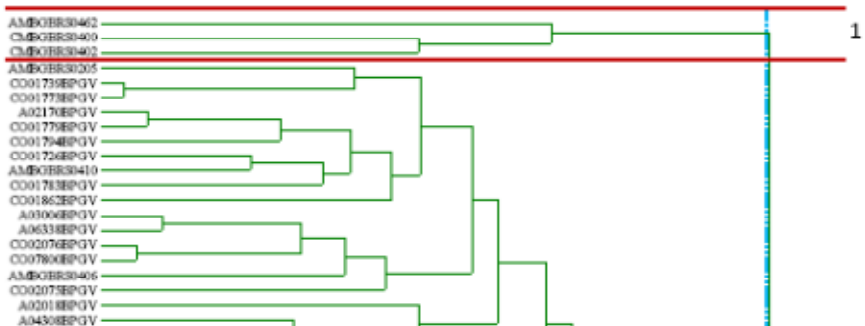


Fig. 4 d by 10 SSR.
ı with acephala



Appendix **4**

Agenda – **Braga Meeting**

Monday, 20 June 2011

Arrival of partners

Tuesday, 21 June 2011

8:30 – 10:00 - BPGV visit, Structure, research, projects

10:00 – 10:30 – Coffee break

10:30 – 12:30 – I**mplementation of the MAA'S in *Brassica oleracea* of Iberian collection: Mode of operation**

- General introduction – Lopes, V. (10 min)
- Selection procedure MAA'S for *Brassica rapa* – Bas N. (20 min)
- Genetically unique within AEGIS – Allender C. (20 min)
- *Brassica* BPGV collection: presentation – Lopes, V. (35 min)
- *Brassica* MBG collection: presentation – Cartea, E. (35 min)

12:30 – 14:00 lunch

14:00 – 16:00 - **Discussion about application of MAA's selection criteria proposed for *B. rapa* to the *B. oleracea* of Iberian collection**

16:00 – 16:30 – coffee break

16:30 – 17:30 – **Proposal and analysis of workplan with identification of next steps of project implementation**

Wednesday, 22 June 2011

Departure of partners

Agenda Meeting AEGIS project: Implementation of the MAA'S in Brassica oleracea of Iberian collection
Pontevedra (Spain), 29 May 2012

Monday, 28 May 2012: Arrival of partners

Tuesday, 29 May 2012

9:00 – 11:00: presentations of different partners
11:00 – 11:30 – Coffee break
11:30 – 13:30 – **Discussion and future plans: Conclusions and final remarks**
14:00 – 16:00 - Lunch
16:30 – 17:30 – MBG visit: labs, field and buildings

Wednesday, 30 May 2012: Departure of partners

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(1)Assessment of genetic diversity in Iberian Landraces of *Brassica oleracea* by molecular markers. Microsoft Word - BRASSICA2012_BPGV.30.06.pdf

Lopes, V.R.[1], Barata, A. M.[1*], Nunes, E. [2], Cartea, E. [3], Soengas, P. [3], Allender, C. [4], Bas, N. [5]

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