MORPHOLOGICAL CHARACTERIZATION OF PORTUGUESE ITALIAN RYEGRASS LANDRACES

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ABSTRACT

Italian ryegrass (Lolium multiflorum Lam.) is one of the most important forage grass species in Europe. In Portugal 34% of the cultivated area is dedicated to animal fodder. Italian ryegrass area in Entre Douro e Minho (EDM) region was mainly with landraces. Changes in traditional agricultural systems are contributing to a major loss of genetic diversity mainly to landraces. Portuguese Italian ryegrass landraces are threatened since nineteen years and collecting missions took place in EDM to ex situ conservation. The main Italian ryegrass landraces from EDM are "castelhano's" and "verdeal's" agro type. The aim of this study was to evaluate the existence of morphological variability between EDM Italian ryegrass landraces, to analyse the existence of duplicates in the germplasm collection and to evaluate the farmer' classification. The landraces were characterised based on morphological traits of International Plant Genetic Resources Institute and International Union for the Protection of New Varieties of Plants descriptors list and the most discriminated traits were utilised in the multivariate analysis, using NTSYS v.2.0.

Italian ryegrass accessions belong to two different agro types based on morphological characteristics and farmers identified. Each landrace showed inter-population variability mainly on "verdeal" landrace. It was possible to differentiate the most of ryegrass accessions using the morphological traits used were initial stage of plant maturity.

The EDM germplasm preserves the Italian ryegrass genetic diversity, which is conserved in ex situ and is a good basis for research programs.

Key words: Italian ryegrass landraces, morphological variability, conservation ex situ

INTRODUCTION

Italian ryegrass is an outcrossing species and is generally observed as a diploid taxon with the chromosome constitution 2n = 2x = 14. It is native to the Po Valley of northern Italy where represents the main grass component of irrigated permanent meadows since the 13th century. It is distributed from Central and Southern Europe to North Africa and to SW Asia. It was introduced in most temperate regions, from the lowlands to about 800 m in the Alps.

The annual ryegrass is one of the most important forage grasses and is widely cultivated in Asia, North and South America, Europe and New Zealand. This species below to a genus Lolium which systematic was studied by Terrel [19, 20] with germplasm collected in Portugal, Spain and Eastern United States. The main centre of diversity for the Festuceae tribe is Eastern Europe, where the genus Lolium is found (Borrill 1976). The origins of annual ryegrass are still undefined but it is know that this species was among the natural vegetation in the fields of northern Italy, where it probably originated [18].

Italian ryegrass (Lolium multiflorum Lam.) and the perennial ryegrass (L. perenne L.) are the most important forage grass species in Europe. They are cultivated for forage and amenity use and as a protection against erosion. These two species cover 23% of the grassland area in Europe [13].

From 1950 to 1956 the area under permanent grassland in Portugal was 1.2% of the total land area. Since then permanent grassland areas steadily increased to 1.39 million of ha for grazing. However, there has been a declined since 2002 to 37.250 hectares (24%) (//agrifish.jrc.it/marsstat/pasture_monitoring/Pask/index.htm; 2/09/08). In Portugal 34% of useable agricultural area (UAA) is main by forage area (www.civ-viande.org/uk/ebn; 15/10/08).

Mountain pastures are an essential component of the agricultural system in the north and centre of Portugal. They are located in Trás-os-Montes, Beira Interior and Entre Douro and Minho. The economic importance of mountain pastures represents 17% of UAA in Trás-os-Montes, 28% in Beira Interior and 19% in Entre Douro e Minho. Mountain pastures are permanent, semi-natural meadows dominated by spontaneous herbaceous plants.

Forage species are significant to promote their conservation. Forage species were collected by Agricultural Minister services from north Portugal during several collecting missions undertaken since 1986 till 2000 in EDM region. The species were overall Lolium multiflorum, Avena strigosa and Trifolium incarnatum landraces and wild or semi-natural populations of Ornithopus ssp., Medicago ssp., Trifolium ssp., Festuca ssp. and Dactylis ssp. Some cereals landraces utilized to animal fodder were collected like Secale cereal and Hordeum vulgare. The seeds were conserved at -18ºC with an accession number and some of them were characterised and evaluated. At this moment most of accessions are conserved in Banco Português de Germoplasma Vegetal (BPGV). One of these species is Italian ryegrass (Lolium multiflorum Lam.) landraces collected mainly from EDM region. The landraces seed samples were collected from fields or farmers' stores.

The Plant Genetic Resources Unit of the Institute of Grassland and Environmental Research, U.K., and the Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro carried out a joint plant collecting expedition in Portugal in May 1995 [6]. One of seven regions of Portugal explored was EDM with support of Agricultural Minister local services. The collect covered different altitudes, management systems and ecological conditions. From EDM region Lolium multiflorum semi-natural populations at arable or grassland habitat and L. hybridum wild and semi-natural populations at diverse habitat (grassland, arable, and forest conifers) were collected.

Italian ryegrass landraces from EDM belong to "castelhano's" and "verdeal's" agro type. Some times the landraces has been identified as "galego" which is an agro type similar to "castelhano's" but much earlier. "Castelhano's" agro type is still intercropped with maize or sowing with others annual species (mainly gramineous) and is characterised as been westerwold with early heading date, low number of tillers/plant, small canopy diameter and low dry matter yield. It is utilized as forage, hay or as silage. "Verdeal's" agro type was utilized generally for grazing, as green or dry forage by multiple cuts (4 harvests). This landrace is characterised by its persistence (2-3 years), late heading date, wider canopy and high tillering capacity (number of tillers/plant), and low or very low tendency to form inflorescences in sowing year. It is a short living perennial [12]. This landrace has not been sowed in the last years in EDM region.

At this moment in Portugal "castelhano" landraces are still intercropped with maize, although seed production is no longer available and, it has been replaced by commercial varieties. Cresswell et al.[7] considered that these changes to traditional agricultural systems are contributing to a major loss of genetic diversity.

The aim of this study was to identify morphological variability among Italian ryegrass landraces from EDM region, to find duplicates in the collection and to evaluate the farmer' classification.

Table 1 Accession numbers in collections, local and year of collection and farmer 'classification to Entre Douro Minho Italian ryegrass germplasm.

BPGV number	Accession number Original Collection	Collect mission	Site	Farmer' Classification
09209	00326	1996	Amarante	verdeal
	00063	1991	Arouca	verdeal
09160	00004	1986	Barcelos	verdeal
09193	00115	1992	Braga	verdeal
	00003	1986	Esposende	verdeal
09203	00237	1995	Esposende	verdeal
09201	00235	1995	Fafe	verdeal
09173	00087	1991	Felgueiras	verdeal
	00088	1991	Felgueiras	verdeal
09174	00089	1991	Felgueiras	verdeal
09163	00009	1986	Maia	verdeal
	00066	1991	Monção	verdeal
09168	00069	1991	Monção	verdeal
	00077	1991	Monção	verdeal
09190	00110	1992	Paços Ferreira	verdeal
	00113	1992	Paços Ferreira	verdeal
04666	00140	1993	Paredes Coura	verdeal
	00002	1986	Penafiel	verdeal
09162	00008	1986	Porto	verdeal
09185	00106	1992	Póvoa Varzim	verdeal
09188	00108	1992	Póvoa Varzim	verdeal
09189	00109	1992	Póvoa Varzim	verdeal
09181	00101	1992	Santo Tirso	verdeal
	00102	1992	Santo Tirso	verdeal
09183	00104	1992	Santo Tirso	verdeal
09184	00105	1992	Santo Tirso	verdeal
09164	00010	1986	V. N. Famalicão	verdeal
09159	00001	1986	Valença	verdeal
	00085	1991	Valongo	verdeal
09186	00099	1992	Valongo	verdeal
09179	00096	1991	Vieira Minho	verdeal
09200	00234	1995	Vieira Minho	verdeal
09162	00005	1986	Vila Conde	verdeal
	00006	1986	Vila Conde	verdeal
	00007	1986	Vila Conde	verdeal
	00011	1986	Vila Conde	verdeal
	00012	1986	Vila Conde	verdeal
09195	00132	1993	Vila Conde	verdeal
09238	00499[20Galiza]	?	Asturias	*Lolium* spp.
09237	00498[4Galiza]	?	Vigo	*Lolium* spp.
04978	00569	1993	Guarda	erva trigal

MATERIAL AND METHODS

The landraces were characterised based on the International Plant Genetic Resources Institute (IPGRI) morphological traits and the UPOV (International Union for the Protection of New Varieties of Plants) descriptors list. The most discriminated traits were utilised on multivariate analysis. The variance analyses and univariate analyse carried out the discriminate descriptors by utilization of MSTAT-C from Michigan University (not showed).

Plant Materials

Ninety two accessions were characterised: 77 EDM landraces, 1 landrace from market, 12 commercial varieties and two semi-natural populations from Galicia

Table 1 Continued.

BPGV number	Accession number Original Collection	Collect mission	Site	Farmer' Classification
	00100	1992	Santo Tirso	castelhano/galego
09180	00098	1992	Valongo	castelhano/galego
09210	00327	1996	Amarante	castelhano
09228	00353	1995	Amares	castelhano
09175	00090	1991	Arouca	castelhano
	00144	1992	Cabeceiras de Basto	castelhano
05799	00500	1994	Cabeceiras de Basto	castelhano
03690	00505	1992	Cabeceiras de Basto	castelhano
03546	00568	1992	Cabeceiras de Basto	castelhano
03569	00572	1992	Cabeceiras de Basto	castelhano
09166	00064	1991	Celorico Basto	castelhano
09204	00238	1995	Esposende	castelhano
09205	00239	1995	Esposende	castelhano
09207	00241	1995	Esposende	castelhano
09202	00236	1995	Guimarães	castelhano
09167	00065	1991	Monção	castelhano
09233	00358	1995	Mondim de Basto	castelhano
03641	00570	1992	Mondim de Basto	castelhano
09191	00111	1992	Paços Ferreira	castelhano
09192	00114	1992	Paços Ferreira	castelhano
09170	00078	1991	Paredes Coura	castelhano
09171	00081	1991	Paredes Coura	castelhano
09236	00495	1998	Paredes Coura	castelhano
09187	00107	1992	Póvoa Varzim	castelhano
09182	00103	1992	Santo Tirso	castelhano
07032	00328	1996	Terras Bouro	castelhano
09196	00133	1993	Vila Conde	castelhano
09206	00240	1995	Vila Conde	castelhano
09208	00242	1995	Vila Conde	castelhano
03965	00567	1992	Vila Real	castelhano
09199	00233	1995	Vila Verde	castelhano
09234	00360	1992	Cabeceiras de Basto	?
05798	00497	1994	Cabeceiras de Basto	?
03701	00506	1992	Cabeceiras de Basto	?
03507	00571	1992	Castelo Branco	?
04881	00503	1993	Celorico da Beira	?
04571	00504	1993	Tondela	?
	00112	1992	Paços Ferreira	"verdeal" introduction from France

(table 1 and 2). The commercial landrace is the castelhano agro type and between commercial varieties two of them are verdeal agro type ("Verdeal", "Bragelim"). Landraces out of EDM region were not identified by farmer (table 1), except "erva trigal" from Guarda. The verdeal agro type was more representative to Monção, Felgueiras, Santo Tirso, Valongo, Vila Conde and Vieira do Minho; castelhano agro type was more frequent in Paredes de

Table 2 Description of commercial varieties utilized as standard.

Commercial varieties	Description
Turilo	Diploid, no westerold
Ninak	Tetraploid, no westerold
Ansyl	Tetraploid, no westerold
Barspectra	Tetraploid, westerold
Liflória	Diploid, westerold
Landras	Diploid, westerold
Verdeal	Diploid, westerold
Liwega	Diploid, westerold
Lirasand	Diploid, westerold
Billion	Tetraploid, westerold
Bragelim	Diploid, westerold
Macho	Hybrid, Tetraploid, no westerold

Coura and Cabeceiras de Basto and both agro types was presented in Esposende and Paços de Ferreira.

Morphological Characterization

Forty to seventy plants per accession were planted at a distance of 0.50 m from each other, laid out in the field in a randomized complete block design, replicated four or five times. Morphological evaluation was obtained in each plant. Each accession were characterised two years to 17 morphological traits. The morphological traits were adapted from the IPGRI descriptor list [9] and UPOV [23] guidelines for the conduct of tests for distinctness, homogeneity and stability. The most discriminated traits, not redundant, were to heading stage: (MHPL) mean height plant (natural height at inflorescence emergence), (LL, WL) length and width leaf (reproductive), (MxHPL) maximum height plant (length of longest stem (including inflorescence) and heading date (HDATE).

Statistical analysis

The most discriminated morphological traits of Italian ryegrass germplasm data were submitted to multivariate analysis using NTSYS v.2.0: the principal component analysis and cluster analysis. For cluster analysis taxonomic distance coefficient was selected as a measure of similarity among all accessions, and the Unweighted pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition.

RESULTS

Morphological variability

The principal component analyse showed that 89 % of total variation of morphological traits was explained by the first three components (table 3). The first one had greater contribution of mean height plant (MHPL), length and width of leaves (LL, WL) and maximum height plant (MxHPL); the second was defined by MxPL (maximum height plant) and heading date (HDATE) and, the third component resulted from linear combination between MHPL (mean height plant), length leave (LL) and HDATE (heading date). The figure 2 illustrates a three-dimensional space defined by three principal' components of landraces and the morphological traits. The first component group tall plants with large leaves; the second differentiated the tall plants with higher length of longest stem (MxHPL) and with late maturity and the third component was to plants with variable length of longest stem (short to long), narrow leaf, heading date and natural height medium.

Verdeal landraces are show in 4 circles in Fig. 2: with short natural height, leaf narrow and lateness (00063, 00066, 00069, and 00088), landraces with natural height medium, leaf narrow and length medium, and lateness (00085, 00096, 00237, 00087, and 00089), landraces semi late, leaf length long and natural height long (00001, 00008, 00009, and 00005), and landraces with natural height long, leaf larger (wide and long) and early heading date (00002, 00006, 00003, 00007, 000012, 00004, 00011). One fifth circle grouped landraces with intermediate maturity, medium leaf and natural height variable (short to medium) but tall longest stem (00064, 00506, 00078, 00234, 00081, 00077, 00570, 00503, and 00235). There were landraces defined by very short plants, short leaf and semi early heading date (00495, 00326, 00504, 4 Galiza and 20 Galiza). Remaining landraces were defined by negative plane between the three principal' components. These landraces were from two agro types, castelhano and verdeal.

The phenogram (figure 3) indicated the pattern of similarities among landraces and classifies the inter-varietal diversity. The morphological traits utilized allowed identify the accessions of landraces and clustered

the agro types into different groups. It was verified that germplasm from same origin presented accessions from two agro types, clustered at different groups.

The accessions of landraces were placed into four main clusters: the group of "verdeal" agro type collect in 1986 (00001 to 00012) characterised by variable heading date (early to semi late), longest stem with length medium, high natural height and long leaf; the second and great group with two agro types represented and clustered into sub-clusters separately; the third group (00063, 00066, 00069 and 00088) was characterised by very late maturity, shorter natural height, shorter longest stem length and very narrow leaf. The last group (00569, "Billion", "Bragelim" and "Macho") clustered overall commercial varieties with an accession which local name ("erva trigal" or herb like wheat) displaed the main characteristic, the higher longest stem. This group describes very tall plants, very late maturity and wide leaves.

In second cluster is marked six sub-clusters: the "verdeal" sub-cluster (00085, 00096, 00237, 00087, 00089); the "castelhano" sub-cluster plus accessions without local name (00065, 00144, 00358, 00572, 00567, 00568, 00497, 00500, 00353, 00505, 00360); the mix of "castelhano" and "verdeal" (from accession 00090 to accession 00571) with the agro types discriminated; the four sub-cluster composed by accession 00099 until accession 00110 and the two sub-clusters with assemble of agro types (accessions from 00064 to 00235 and the other with accessions from 000495 to accession 00328).

The "verdeal" agro type was collected overall in beginning of nineteen years and the "castelhano" agro type was collected since 1991 to 1996. However the variability to "castelhano" agro type was less significant. The accessions of "castelhano" agro type displayed less variability inter-population. The collect year and origin were not source of spatial and temporal diversity to discriminate accessions. Nevertheless it was possible to observe the "castelhano" accessions from Basto region were clustered at same group.

The "verdeal" agro type showed more variability between its accessions. Theirs accessions were grouped by collect year and two groups were in evidence by origin: the group of "verdeal" accessions from S.Tirso and P.Varzim and the group which "verdeal" accessions were from interior region (Monção, Arouca Felgueiras). The accessions of "verdeal" agrotype from interior region showed more variability between them. The "verdeal" accessions from littoral region were less diverse of "castelhano" accessions from this region.

The accessions of two agro types were differentiated from commercial varieties. Although some of these commercial varieties ("Barspectra", "Liflória", "Landras", "Verdeal", "Liwega" and "Lirasand") were clustered at same great group with mostly of accessions.

The phenogram showed the same pattern of variation like it was explained by principal components analysis. The landraces with characteristics more vegetative vigour (plants tall, with large leaf) and lateness were clustered. There is genetic variability with genetic distance coefficient 2.08 which coefficient correlation cophonetic was 83%.

Duplicates in collection

The duplicates were analysed considering the phenogram (Fig.3). The "verdeal" accessions more similar were 00002, 00006 and 00003, 00007. It is possible considering duplicates in the case of "verdeal" accessions 00104, 00106 and 00108, 00110. In "castelhano" accessions there were not duplicates but some accessions were linked like the accessions 00090, 00241 and 00239, 00240. The "galego" was clustered at same group and were very similar.

Farmer' classification

The accession 00112 was incorrectly designated by farmer as "verdeal" but the farmer ex émigré brought it from France and the accession was perfectly isolated. The accession 00569 with a specific local name and out from EDM was too isolated from EDM accessions and placed with commercial variety "Billion".

The "verdeal" accession 00113 was placed nearby to "castelhano" accession 00100 and their origin was not far from. The same takes place among accessions 00132 ("verdeal"), 00238 ("castelhano") and 00495 ("castelhano"), 00326 accession ("verdeal"). Some accessions (00078, 00234, 00081, 00077, 00570) with different designations were placed around however were differentiated.

The observation of phenograme allows considered that EDM farmers differentiated clearly the two agro types then designated distinctly theirs accessions.

DISCUSSION

Portugal in particular Entre Douro e Minho is a region characterised by favourable climatic conditions to temperate grasses, where Italian ryegrass had good situation to distribute, to adapt and following it was utilized by farmers. Already in 1983 the Italian ryegrass was considered one of most interesting forage species by occupied larger areas from Portugal what was a motivation to obtain commercial varieties from ecotypes and national populations [17]. Two commercial varieties ("Bragelim", "Verdeal") utilised in this study are the example for this purpose.

Table 3 Contribution of morphological traits to first three components obtained by principal component analyse, which showed 89% of total variation explained by 3 components.

	Eigen value	Percent	Percentage Cumulative			
1	2.48857683	49.77	49.77			
2	1.26143856	25.22	75.00			
3	0.74928693	14.98	89.98			
				1	2	3
MHPL				0.8576	0.0285	0.3204
WL				0.8642	-0.2157	-0.2574
LL				0.8878	-0.0596	0.2872
MxHPL				0.4596	0.6310	-0.6017
HDATE				-0.0834	0.9013	0.3685

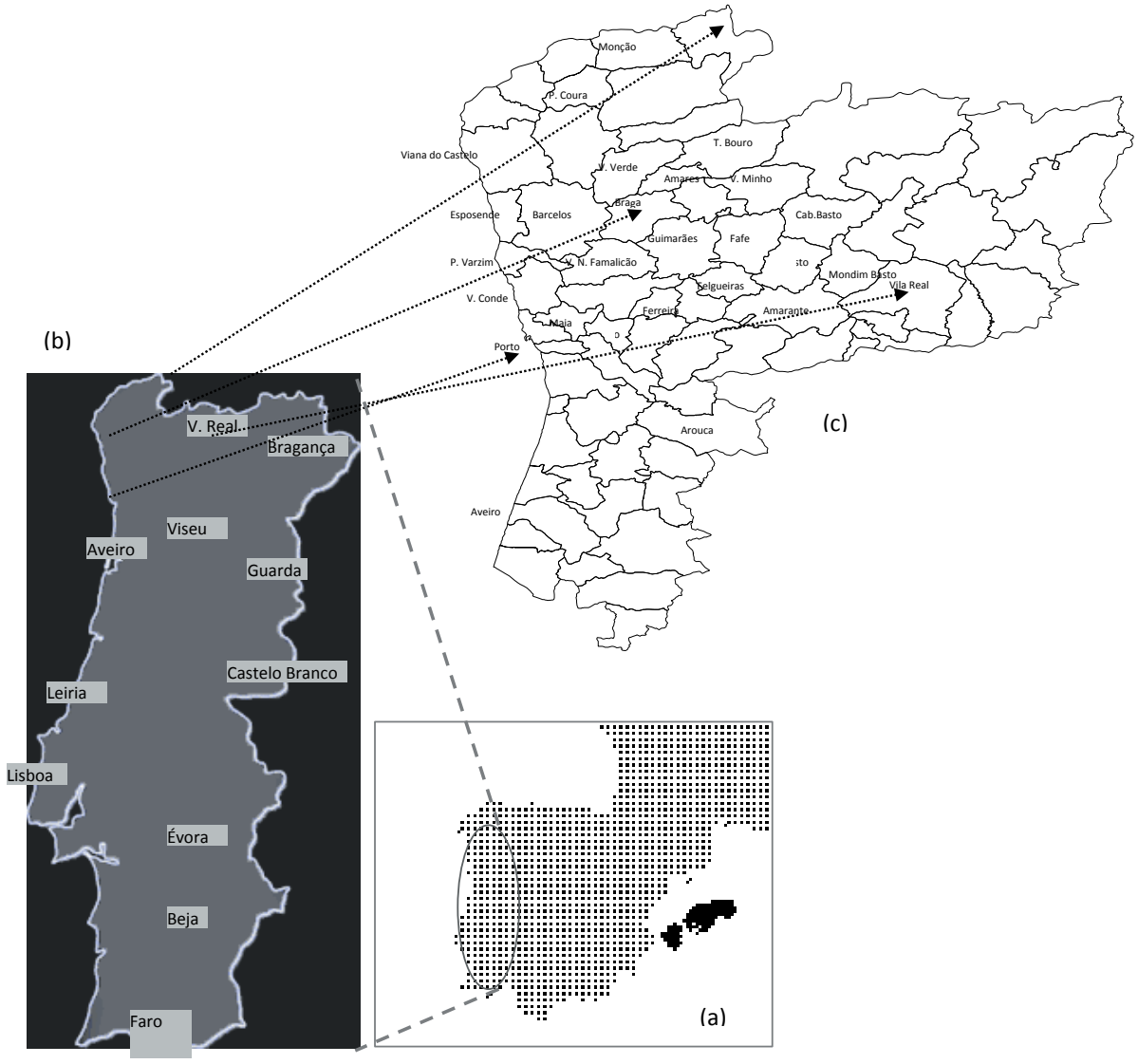

Figure 1 Origin of Italian ryegrass germplasm collection: the Peninsula Iberia map (a); Portugal map (b); Entre Douro Minho map (c).

The improvement of this crop is important. When defining the breeding and conservation strategies to be put in place we must know the degree of genetic diversity. This research contributes to this goal.

The morphological traits at initial stage of plant maturity were useful to identify and differentiate accessions. The plant maturity is the main factor that influences forage quality and the grow characteristics of this stage are important to yield.

The EDM Italian ryegrass accessions are from two different agro types whose morphological characteristics differentiate. They are landraces how Camacho Villa et al. [3] proposed as definition: landrace is a dynamic population(s) of a cultivated plant that has historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems. Also these landraces showed being dynamic populations. Each landrace showed inter-population variability to morphological traits, more significant to "verdeal" landrace. The variability displayed influence of ecogeographics conditions and of farmer decision. The

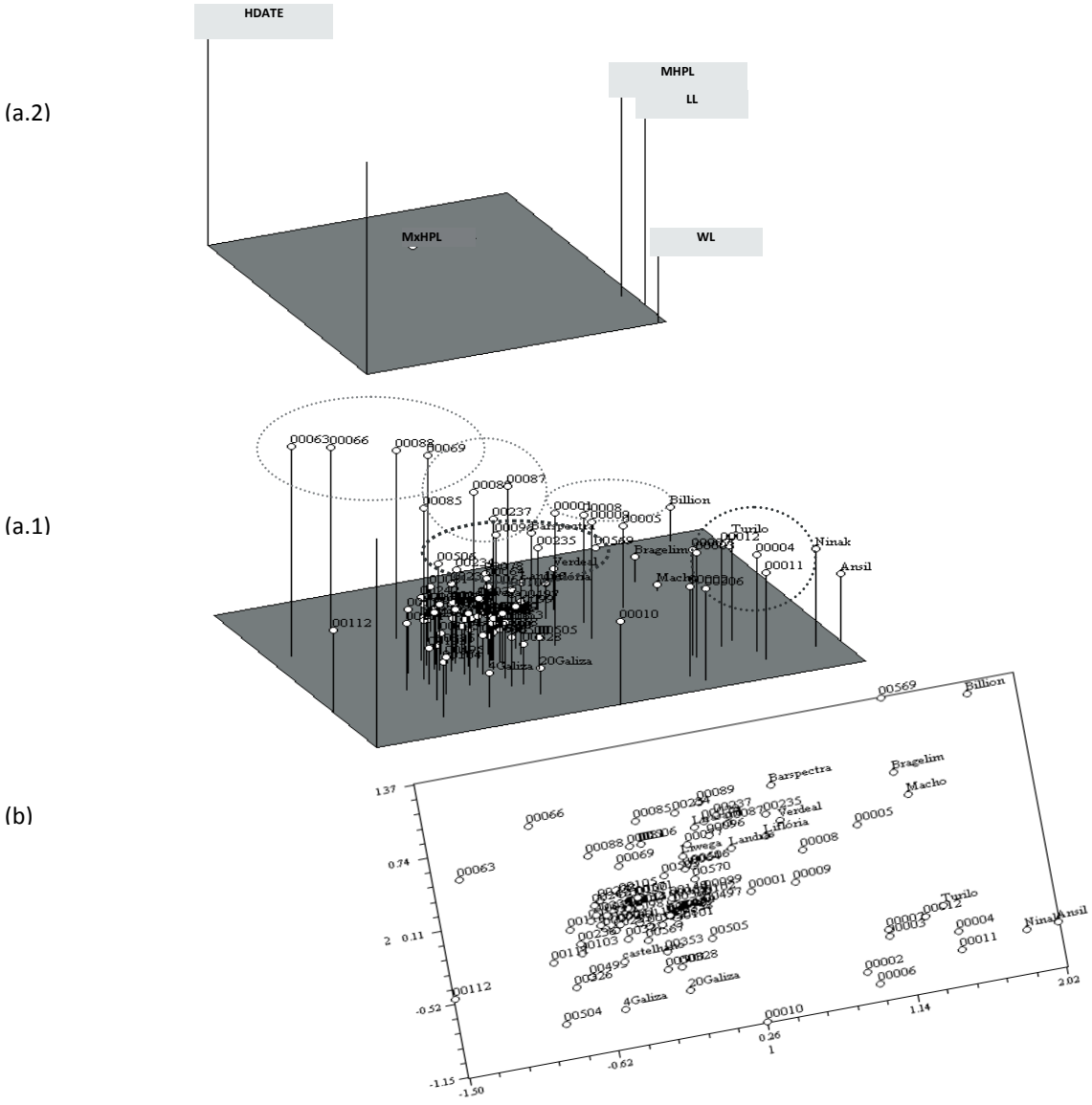

Figure 2 Projection of Italian ryegrass landraces (a.1) and morphological traits (a.2) on three-dimensional space defined by first three components and, Italian ryegrass landraces' projection on bi-dimensional space (b) defined by two principal components which explained 75% of total variation.

accessions or populations from each landrace adapted to local conditions, evolved and developed characteristics resulting from agricultural systems (intercrop or pastures) and how farmer managed them (forage conserved hay or silage, one or multiples cuts…, seed production) as Hayward et al. [8], Balfourier & Charmet [2], Charmet et al. [4, 5], Loos [10,11], Ursla et al. [24] found to perennial ryegrass populations differentiation. Intercrop system (ryegrass and maize intercrops) can promote earliness, narrow leaf; hay production can originated populations highest and earliest, multiples cuts or grazing can make populations with long leaf and latest. These relationships were found by several researchers to perennial ryegrass [4, 11, 12, 14, 15, 16, 24]. EDM Italian ryegrass landraces shows these relationships: "castelhano" intercrops with maize is more earliness and theirs leaves narrower; "verdeal" utilized to multiples cuts is latest and leaves are great. The ecogeographic variation determines the inter-population differentiation evolving to adaptive traits and the men promoted the agro diversity. Besides, the natural introgression and natural hybridations between perennial ryegrass and Italian ryegrass were already mentioned by Tyler et al. [22] that considered the entire European population was a group of hybrids with continuous variation and the two species in the extremes: perenne types dominating the heavily grazed areas and multiflorum types in hay meadows. Tobina et al. [21] found hybridization between perennial ryegrass and Italian ryegrass in naturalized Japanese populations. This description is agreed with presence of Lolium x hybridum Hausskn in Portuguese natural populations. In the wild, their distribution is in the area common to the two parents.

Chorlton et al. [6] collected in EDM 24 wild or semi-natural Lolium x hybridum populations and 7 semi-natural L. multiflorum populations. The wild or semi-natural L. perenne populations were found out from EDM but in North (Trás-os-Montes) too. Cresswell et al. [7] investigated with AFLP (amplified fragment lengh polymorphism markers) the same collect to assess genetic diversity of Lolium species from Portugal. Some multiflorum (2) and hybridum (4) accessions were from EDM and they were genetically related excepted to one of hybridum group. The perenne group were clustered discrete widely separated from all the other populations. This result is in contrast with morphological studies of Tyler et al. [22]. The continuous variation with natural hybridations was not verified.

To know the genetic relationships among EDM landraces and between them and Lolium x hybridum and L. perenne it will be necessary more and different studies, like molecular studies. However, it is expectable the importance of natural crossing among L. x hybridum and L. multiflorum species. This natural crossing will can justify the characteristics of "verdeal" landrace as been a short living perennial with low tendency to form inflorescences when not exposed to short day or low temperature vernalization. Cresswell et al. [7] found the two species at arable and grassland habitat what facilities the natural crossing.

EDM germplasm preserves the Lolium spp. genetic diversity, which is conserved in BPGV collection and is a good basis for research programs. Though in risk, the "castelhano" landrace is still grow in association with maize, which is not happening with "verdeal" landrace. This landrace was not saved within traditional or subsistence farming system, but eradicated by replacement with modern cultivars. These two would be the recommended landraces to integrate the regional on-farm programme or a national programme.

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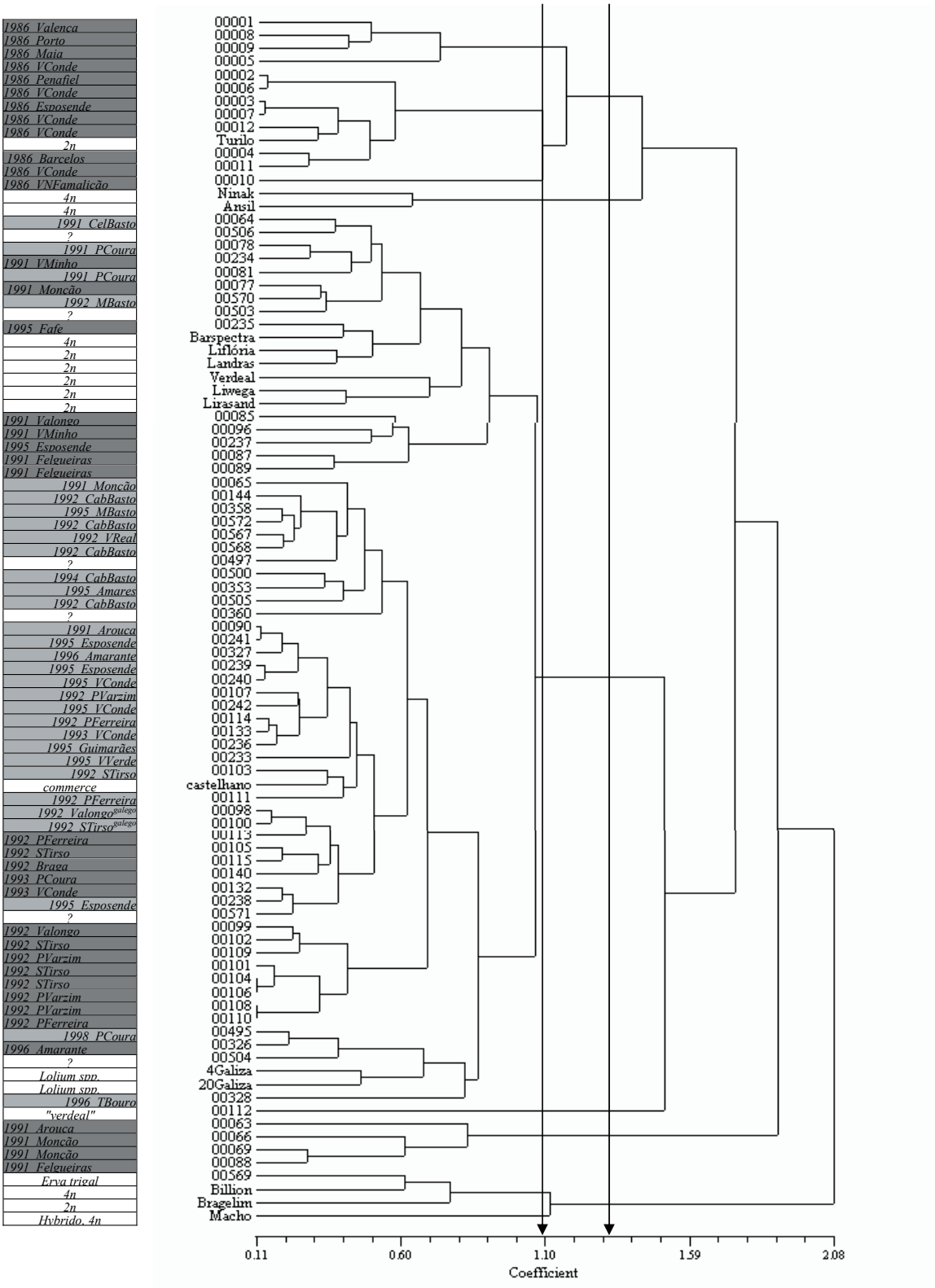

method (collect' year, origin and identification of landraces from phenogram: "castelhano" landrace by grey shade and "verdeal" landrace by dark grey shade; two arrows define clusters to different genetic distance coefficient).