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Research Article

Iberian Peninsula cowpea diversity: chloroplast, microsatellite and morpho-agronomic variability

ELIANA MONTEIRO[1], ISAURA CASTRO[1,2], MÁRCIA CARVALHO[1], JUAN PEDRO MARTÍN[3], EDUARDO ROSA[1,4] & VALDEMAR CARNIDE[1,2]

[1]Centro de Investigação e de Tecnologias Agro-ambientais e Biológicas (CITAB), Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real 5000-801, Portugal
[2]Departamento de Genética e Biotecnologia, Escola de Ciências da Vida e do Ambiente, Universidade de Trás-os-Montes e Alto Douro, Vila Real 5000-801, Portugal
[3]Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Departamento de Biotecnología-Biología Vegetal, Universidad Politécnica de Madrid, Avda. Puerta de Hierro, Madrid 28040, Spain
[4]Departamento de Agronomia, Escola de Ciências Agrárias e Veterinárias, Universidade de Trás-os-Montes e Alto Douro, Vila Real 5000-801, Portugal

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Cowpea (*Vigna unguiculata* (L.) Walp.) is an important legume crop in Southern Europe and a valuable source of proteins, minerals, and vitamins. Moreover, cowpea has additional interest stemming from drought tolerance and high biological nitrogen fixation. In this research, the genetic diversity of cowpea landraces from Southern European countries was evaluated using molecular and morpho-agronomical approaches, with the objective of enhancing legumes diversity grown through sustainable cropping. A set of 10 chloroplast microsatellite primer pairs (cpSSRs) was used to evaluate genetic diversity and phylogenetic relationships among 113 *Vigna* accessions including *Vigna unguiculata* subsp. *unguiculata*, subsp. *alba,* subsp. *pubescens,* subsp. *tenuis* and var. *spontanea* and accessions from other *Vigna* species, as *V. mungo*, *V. radiata* and *V. racemosa*. This set of primers successfully established ten haplotypes, with the most frequent being shared by the *V. unguiculata* subsp. *unguiculata* cultigroups unguiculata and sesquipedalis and var. *spontanea.* Eight loci were polymorphic; nevertheless, a low level of polymorphism was verified within the cultivated cowpeas. Additionally, 10 agronomic traits were evaluated on cowpea landraces of the cultigroup unguiculata, mainly from Portugal. PCA clustered the landraces into three main groups, each one containing Portuguese landraces. Total seed weight per plant revealed the highest coefficient of variation, and 100 seed weight the highest heritability. The performed study shows the wide agro-morphological diversity still existing in cowpea in Iberian Peninsula and other Southern European countries, despite the low polymorphism detected in its chloroplast genome. The high variability detected in the collection of cowpea analysed and the sharing of haplotypes by cultivated and wild material is of great importance for breeding programs of this species.

Key words: agro-morphological diversity, cpSSR, germplasm, haplotype, Iberian gene pool, landraces, legumes, molecular markers

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a diploid species (2 n = 2 x = 22) native to Africa. This grain legume possesses a high protein content, which varies between 26%–28% in green leaf and 23%–32% in seeds (Gonçalves et al., 2016; Iqbal et al., 2006; Tan et al., 2012). It can naturally improve soil fertility due to its ability to fix atmospheric nitrogen through symbiosis with nodular bacteria (*Bradyrhizobium* spp.) (Kalloo & Bergh, 1993; Singh et al., 1997; Valenzuela & Smith, 2002). Cowpea is able to tolerate fluctuating climatic conditions and is thought to be drought tolerant, characteristics which may help this species to adapt in a changing climate (Agbicodo et al., 2009; Ehlers & Hall, 1997; Hall, 2012; Kotze, 2015).

Vigna unguiculata L. has 11 subspecies, only one of which is annual (subsp. *unguiculata*), comprising wild (var. *spontanea*) and cultivated (var. *unguiculata*) forms (Vijaykumar et al., 2012). All cultivated cowpeas are

Correspondence to: Isaura Castro. E-mail: icastro@utad.pt

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grouped under *V. unguiculata* subsp. *unguiculata* and sub-divided into four cultigroups, being unguiculata and sesquipedalis cultigroups the most used in human food (Coulibaly et al., 2002; Ehlers & Hall, 1997).

Genetic diversity is an important research area as the accurate assessment of genetic variability is useful for the preservation and utilization of germplasm resources and to improve varieties/cultivars (Tan et al., 2012). Several studies report the characterization of cowpea by morphological and agronomic traits (Adewale et al., 2011; Cardona-Ayala et al., 2013; Pasquet, 1998; Stoilova & Pereira, 2013). Recently, there has been a focus on the characterization of southern European cowpea germplasm from Greece (Lazaridi et al., 2017), Italy (Lioi et al., 2019) and the Iberian Peninsula (Carvalho et al., 2016, Carvalho, Bebeli, et al., 2017; Martos-Fuentes et al., 2017) as the Southern Europe climate conditions are adequate to its production and this grain legume is much consumed in this region. Southern European cowpeas are considered important nutritional and environmental resources, well adapted to their agro-system, and with a natural tolerance for water scarcity conditions and high temperatures, though they should be genetically preserved and improved for their efficient use (Carvalho, Lino-Neto, et al., 2017; De Ron et al., 2018). At the level of genetic diversity, there have thus far been few studies of cowpea using material from Europe and the Iberian Peninsula, as in our study, and the most recent study uses SNP (Carvalho, Muñoz-Amatriaín, et al., 2017). Molecular markers based on PCR to detect chloroplast DNA polymorphisms, such as microsatellites (cpSSRs), are a powerful tool for studies of genetic diversity and network and phylogenetic inference. This marker has been widely used for genetic studies in other crops (Castro et al., 2013, 2019; Desiderio et al., 2013; Ferreira et al., 2015; Herrera et al., 2008; Powell et al., 1996). Knowledge of the variability and evolution in the chloroplast genome of *V. unguiculata* is limited at the molecular level (Provan et al., 2001). After pairs of consensus primers were developed in a diverse set of chloroplast genomes including cowpea by Pan et al. (2014) and a complete sequence of the chloroplast genome *Vigna unguiculata* L. was submitted to GenBank by Aragon and Motta in 2012 (NCBI Reference Sequence NC_018051.1), it became possible to begin studying the chloroplast genome using cpSSRs, as in the species mentioned above.

The main objective of this study was to evaluate genetic diversity in a collection of Southern European cowpea, focusing on the Iberian Peninsula germplasm. In order to situate the Iberian cowpea landraces germplasm in the context of worldwide *Vigna* chloroplast diversity a cpSSR haplotype was established in a set of *Vigna* accessions, including *Vigna unguiculata* subsp. *unguiculata*, subsp. *alba,* subsp. *pubescens,* subsp. *tenuis* and var. *spontanea* and accessions from other *Vigna* species, as *V. mungo*, *V. radiata* and *V. racemosa*, preserved in eight different germplasm collections; and then, the agro-morphological performance of 42 Iberian cowpea landraces was evaluated, along with 8 landraces from southern Europe, namely Italy and Greece.

Materials and methods

Plant material

In this study 113 *Vigna* accessions were analysed (Table 1), comprising: −66 *Vigna unguiculata* subsp. *unguiculata* accessions from the Iberian Peninsula (around 80% from the cv-gr. unguiculata); −34 accessions of *V. unguiculata* subsp. *unguiculata* from 18 countries worldwide, −8 accessions of the subspecies *alba, pubescens, tenuis* and var. *spontanea* and −5 accessions of the *Vigna* species *mungo*, *radiata* and *racemosa*. This material was conserved in eight germplasm banks, namely the National Institute for Agrarian and Veterinarian Research (INIAV, Elvas, Portugal), the National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA, Alcalá de Henares, Spain), the Germplasm Bank of Horticultural Species of the Center for Agro-Food Research and Technology (BGHZ-CITA, Zaragoza, Spain); the Agricultural University of Athens (AUA, Greece); the Institute of Biosciences and Bioresources of the Italian National Research Council (IBBR-CNR, Bari, Italy); the Botanic Garden Meise (Belgium); the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany) and the Brazilian Agricultural Research Corporation (EMBRAPA, Brazil).

Chloroplast SSRs amplification

A total of 113 accessions was analysed using cpSSRs markers (Table 2). For each accession, young and healthy leaves, of about 4 cm, were collected and stored at −80 °C until use. For DNA extraction, the leaf tissues were disrupted using the TissueLyser equipment (Qiagen, Chatsworth, USA) and DNA purified using the plant DNA extraction kit NucleoSpin Plant II (Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's instructions.

A total of twenty-one primer pairs of chloroplast SSR primers were selected for a preliminary analysis from the bibliography (Weising & Gardner, 1999; Chung & Staub, 2003; Pan et al., 2014). This selection was based on the polymorphism obtained in those studies and the distribution of each loci throughout the 11 cowpea

Table 1. *Vigna* accessions analysed in the cpSSR study.

Accession number	Bank code[a]	Cultigroup/Species	Locality/Country	Common name
Vg160	Faial	sesquipedalis	Açores, Faial/Portugal	Feijão a metro
Vg11	Vg11	unguiculata	Torre de Moncorvo/Portugal	Feijão frade
Vg13	Vg13	unguiculata	Alijó/Portugal	Feijão frade
Vg47	Vg47	unguiculata	Almeida/Portugal	Feijão frade
Vg97	CP5648	unguiculata	Abrantes/Portugal	Feijão frade
Vg99	CP5651	unguiculata	Ponte de Sor/Portugal	Feijão frade
Vg69	Vg69	unguiculata	Bragança/Portugal	Feijão frade
Vg158	CPS-8	sesquipedalis	Bragança/Portugal	Feijão a metro
Vg64	Vg64	unguiculata	Celorico da Beira/Portugal	Feijão frade
Vg62	Vg62	unguiculata	Covilhã//Portugal	Feijão frade
Vg88	CP4924	unguiculata	Évora/Portugal	Feijão frade
Vg86	CP4847	unguiculata	Ferreira do Alentejo/Portugal	Feijão frade
Vg48	Vg48	unguiculata	F. Castelo Rodrigo/Portugal	Feijão frade
Vg59	Vg59	unguiculata	Fundão/Portugal	Feijão frade
Vg91	CP5128	unguiculata	Lardosa/Portugal	Feijão frade
Vg56	Vg56	unguiculata	Macedo de Cavaleiros/Portugal	Feijão frade
Vg51	Vg51	unguiculata	Meda/Portugal	Feijão frade
Vg15	Vg15	unguiculata	Miranda do Douro/Portugal	Feijão frade
Vg18	Vg18	unguiculata	Mirandela/Porugal	Feijão frade
Vg72	Vg72	unguiculata	Mogadouro/Portugal	Feijão frade
Vg95	CP5556	unguiculata	Mértola/Portugal	Feijão frade
Vg58	Vg58	unguiculata	Penamacor/Portugal	Feijão frade
Vg49	Vg49	unguiculata	Pinhel//Portugal	Feijão frade
Vg87	CP4906	unguiculata	Ansião/Portugal	Feijão frade
Vg96	CP5647	unguiculata	Gavião/Portugal	Feijão frade
Vg12	Vg12	unguiculata	Bragança/Portugal	Feijão frade
Vg60	Vg60	unguiculata	Sabugal/Portugal	Feijão frade
Vg94	CP5553	unguiculata	Sertã/Portugal	Feijão frade
Vg104	CP5554	unguiculata	Sousel/Portugal	Feijão frade
Vg52	Vg52	unguiculata	Trancoso/Portugal	Feijão frade
Vg54	Vg54	unguiculata	Valpaços/Portugal	Feijão frade
Vg101	CP5645	unguiculata	Vila Nova de Ourém/Portugal	Feijão frade
Vg85	CP5263	sesquipedalis	Portugal	Feijão frade
Vg252	Vg252	unguiculata	Baião/Portugal	Feijão frade
Vg245	BGE028976	unguiculata	Albacete, Yeste/Spain	Ciriguello
Vg230	BGE043764	unguiculata	Alicante, Lorcha/Spain	Careto
Vg251	BGE031003	unguiculata	Avila, Candeleda/Spain	Carilla
Vg222	BGE024703	unguiculata	Baleares, Palma de Mallorca/Spain	Fesol
Vg227	BGE040818	sesquipedalis	Cadiz, Jerez de la Frontera/Spain	Habichuela de verdeo
Vg228	BGE040819	sesquipedalis	Cadiz, Zahara/Spain	Chicharo
Vg240	BGE039238	sesquipedalis	Cordoba, Baena/Spain	Judia antigua
Vg249	BGE039237	unguiculata	Cordoba, Baena/Spain	Higuelo
Vg236	BGE035391	unguiculata	Badajoz, Garlitos/Spain	Carilla
Vg244	BGE035390	unguiculata	Badajoz, Oliva de la Frontera/Spain	Frailiño careto
Vg223	BGE025201	unguiculata	Caceres, Villanueva de la Vera/Spain	Carilla
Vg224	BGE025213	unguiculata	Caceres, Arroyomolinos de la Vera/Spain	Minine
Vg217	BGE019751	unguiculata	Gerona, La Bisbal d'Emporda/Spain	Frijol d'hiver
Vg220	BGE022147	unguiculata	Granada, Portugos/Spain	Friguelo
Vg247	BGE040000	sesquipedalis	Granada, Cortes de Baza/Spain	Habilla
Vg239	BGE036461	unguiculata	Huelva, Villanueva de los Castillejos/Spain	Carilla
Vg191	NC105325	unguiculata	Huesca, Fraga/Spain	Ojo de perdiz
Vg192	NC105327	sesquipedalis	Huesca, Ballobar/Spain	Metrera
Vg229	BGE041751	sesquipedalis	Jaen, Albanchez de Magina/Spain	Habicholon
Vg241	BGE039236	unguiculata	Jaen, Castillo de Locubin/Spain	Jiguelo
Vg237	BGE038476	sesquipedalis	Malaga, Alhaurin el Grande/Spain	Habichuela larga
Vg243	BGE038474	unguiculata	Malaga, Genalguacil/Spain	Chicharo
Vg226	BGE027108	sesquipedalis	Murcia, Mula/Spain	Bisuelo
Vg84	Vi4	sesquipedalis	Murcia/Spain	
Vg231	BGE044375	unguiculata	Orense, Cenlle/Spain	Xudia
Vg212	BGE002195	unguiculata	Orense, Lobios/Spain	Carilla

(*continued*)

Table 1. Continued.

Accession number	Bank code[a]	Cultigroup/Species	Locality/Country	Common name
Vg232	BGE047731	unguiculata	Pontevedra, Arbo/Spain	Cajabicho
Vg250	BGE037805	sesquipedalis	Sevilla, Casariche/Spain	Habichuela larga
Vg221	BGE024406	sesquipedalis	Tarragona, Riudecanyes/Spain	Judia
Vg235	BGE036462	unguiculata	Valencia, Carcaixent/Spain	Judia careta
Vg248	BGE040426	unguiculata	Zamora, Asturianos/Spain	Carilla
Vg190	NC105329	sesquipedalis	Zaragoza, Barrio Oliver/Spain	Judia larga
Vg138	NI 206	unguiculata	Angola	–
Vg152	–	unguiculata	Brazil	Miudo Preto Aparecido
Vg154	–	unguiculata	Brazil	Nordeste
Vg155	–	unguiculata	Brazil	Miudo Mamoninha
Vg156	–	unguiculata	Brazil	Baio Coofam
Vg28	A4 E 007	unguiculata	Bulgaria	–
Vg29	A4 E 008	unguiculata	Bulgaria	–
Vg32	Vg 87210026	unguiculata	Bulgaria	–
Vg34	Vg 95210023	unguiculata	Bulgaria	–
Vg125	VIG 10	unguiculata	China	–
Vg144	NI 1183	unguiculata	China	–
Vg151	NI 262	sesquipedalis	China	–
Vg140	NI 22	unguiculata	D.R. Congo	–
Vg137	VIG 206	unguiculata	Cuba	–
Vg117	VIG 66	unguiculata	Egypt	–
Vg116	VIG 90	unguiculata	Egypt	–
Vg118	VIG 71	unguiculata	Ghana	–
Vg161	AUA1	unguiculata	Greece	–
Vg162	AUA2	unguiculata	Greece	–
Vg208	MG 106823	unguiculata	Greece	Mavromatica
Vg209	MG 107571	unguiculata	Greece	Lianofasula
Vg146	NI 778	unguiculata	India	–
Vg147	NI 784	unguiculata	India	–
Vg127	VIG 1650	unguiculata	Iran	–
Vg130	VIG 100	unguiculata	Iraq	–
Vg187	5426	unguiculata	Italy	–
Vg193	MG 115107	unguiculata	Italy	–
Vg204	MG 113779	unguiculata	Italy	Fagiolini pinti baresi
Vg206	MG 112248	unguiculata	Italy	–
Vg132	VIG 87	unguiculata	Libya	–
Vg142	NI 1139	unguiculata	Madagascar	–
Vg159	–	unguiculata	Nigéria	–
Vg120	VIG 49	unguiculata	Senegal	–
Vg123	VIG 51	unguiculata	Zambia	–
Vg259	NI 1656	subsp. *alba*	Angola	–
Vg264	NI 1754	subsp. *alba*	D. R. Congo	–
Vg260	NI 989	subsp. *pubescens*	Kilifi distr., Whispering Palms Hotel/ Kenya	–
Vg262	NI 1862	subsp. *pubescens*	Korogwe/Tanzania	–
Vg257	NI 1655	var. *spontanea*	Diégo Suarez, Antsakoafe/Madagascar	–
Vg254	NI 963	var. *spontanea*	Casamance, Cap Shirring/Senegal	–
Vg256	NI 1808	subsp. *tenuis*	Inhaca Island/Mozambique	–
Vg263	NI 1664	subsp. *tenuis*	Luanshya–Mpongwerd/Zambia	–
Vg175	NI 207	*Vigna mungo*	Kasaï, INEAC Gandajika/D.R. Congo	–
Vg168	NI 635	*Vigna mungo*	Maharashtra, Khandala/India	–
Vg170	NI 239	*Vigna racemosa*	Kasaï, INEAC Gandajika/D.R.Congo	–
Vg179	NI 977	*Vigna racemosa*	Wuga-Murestird/Nigéria	–
Vg184	NI 159	*Vigna radiata*	Accra/Ghana	–

Origin of the Iberian Peninsula and other countries cultivated *V. unguiculata* subsp. *unguiculata*, cultigroups unguiculata and sesquipedalis and of the accessions of wild *V. unguiculata* subsp. *unguiculata* var. *spontanea*, other *V. unguiculata* subspecies and other *Vigna* species studied.

[a]Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; NC, Centro de Investigación y Tecnología Agroalimentaria. Banco de Germoplasma de Hortícolas, Zaragoza, Spain; AUA, University of Athens, Athens, Greece; MG, Institute of Biosciences and Bioresources (IBBR), Italian National Research Council (CNR), Bari, Italy; NI, Botanic Garden Meise, Belgium; VIG, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Germany.

Table 2. Sequence, labelling, position in the cowpea genome, expected and observed size and annealing temperature of amplification of the 10 cpSSR primer pairs used.

Locus	Repeat motif	Primer sequence	Dye	Position in cowpea[d]	Location/region[d]	Expected size (bp)[d]	Observed alleles (bp)	Annealing temperature (°C)
VgcpSSR1[a]	(TA) 5	F: GGTGGATGTTTATACCCAATCG R: TCTTTCTGCGATACAAACAAGAA	NED	7256-7277 7481-7503	*trnK-rbcL* IGS LSC	248	248 249 259 262	55
VgcpSSR10[a]	(AT) 5	F: GGGCTCATTGGCTGTAGAAA R: CCATCTCTCCCCAATTGAAA	PET	55732-55751 55876-55895	*trnR-trnS* IGS LSC	164	166 167 184 193 194	56
VgcpSSR12[a]	(AT) 6	F: GGCCATTTATCCCACTTTCC R: CCAGTCTCTACTGGGGGTTA	PET	64456-64475 64686-64705	*psbJ-psbL-psbF* IGS LSC	250	236 238 240 251	56
VgcpSSR14[a]	(AT) 5	F: TGGATCATAATCCTTGAACATCA R:TGCGAAAACAAAGATAAGAAATCA	VIC	113630-113652 113814-113836	*PsaC-ndhE* IGS SSC	208	207 216 223 231 233	59
ccSSR4[b]	(T)8	F: AGGTTCAAATCCTATTGGACGCA R:TTTTGAAAGAAGCTATTCARGAAC	VIC	54258-54236 53997-54020	*TrnR-AtpA* LSC	≈262	256 258 263 276 277	50
ccSSR7[b]	(T)11	F: CGGGAAGGGCTCGKGCAG R: GTTCGAATCCCTCTCTCTCCTTTT	FAM	28742-28725 28438-28461	*PsbC-TrnS* LSC	≈205	304 312 313	50
ccSSR22[b]	(T)8	F:CCGACCTAGGATAATAAGCYCATG R: GGAAGGTGCGGCTGGATC	FAM	132219-132242 132381-132398	*TrnL-16SrRNA* LSC	180	179	53
ccmp3[c]	(T)11	F: CAGACCAAAAGCTGACATAG R: GTTTCATTCGGCTCCTTTAT	FAM	54775-54755 54693-54712	*trnG intron* LSC	≈83	79 84	50
ccmp7[c]	(A)13	F: CAACATATACCACTGTCAAG R: ACATCATTATTGTATACTCTTTC	NED	9938-9919 9789-9812	*atpB-rbcL* IGS LSC	≈150	146 147 148 149	50
ccmp10[c]	(T)14	F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGTAAATAG	NED	150400-150379 150304-150323	*rpl2-rps19* LSC	≈97	94	50

[a]Pan et al. (2014).
[b]Chung and Staub (2003).
[c]Weising and Gardner (1999).
[d]Position and expected size according to primers homology with the published *V. unguiculata* L. complete chloroplast genome sequence available in Genebank (accession NC 018051).

linkage groups. Of these markers, 10 were selected based on the best amplification results and polymorphism in the material of this study. The original consensus primers were used, as these were PCR efficient without the need for sequence modification for the *Vigna unguiculata* chloroplast genome sequence. For each of the 10 cpSSR loci analysed the forward primer was fluorescently labelled (Table 2). The amplifications were carried out separately for each cpSSR *locus* in a thermal cycler (Biometra, Göttingen, Germany) and PCR conditions were optimized based on the protocols of Weising and Gardner (1999), Chung and Staub (2003) and Pan et al. (2014). The amplifications were performed in a final volume of 20 µL. For ccmp primers (Weising & Gardner, 1999), the reaction mixture contained: 1 × *Taq* buffer, 0.025 mg BSA, 10 ng of genomic DNA, 2 mM MgCl2, 0.15 µM dNTPs, 0.4 µM for each primer, and 0.175 U *Taq* polymerase (NzyTech Lisbon, Portugal). For ccSSR primers (Chung & Staub, 2003) and VgcpSSR primers (Pan et al., 2014), the reaction mixture contained: 1 × *Taq* buffer, 0.025 mg BSA, 10 ng of genomic DNA, 2.5 mM MgCl2, 0.2 µM dNTPs, 0.5 µM for each primer, and 0.05 U *Taq* polymerase (NzyTech, Lisbon, Portugal). PCR products were revealed by electrophoresis on 2.5% agarose gels (w/v) and run for 1 hr at a constant voltage of 150 V. Dilutions of the PCR products were run on the ABI Prism® 3730 Genetic Analyzer using the GeneScan™500 LIZ® size standard (PE Applied Biosystems, Foster City, CA, USA).

Labelled products of cpSSRs were analysed and sized using Peak Scanner™ v1.0 free software (PE Applied Biosystems, Foster City, CA, USA). Data analysis of cpSSR amplicons was performed by means of GenAlEx 6.5 software to determine allele frequency, the number of effective alleles (*Ne*), using the following equation: $Ne = 1/\Sigma pi2$. Shannon's information index (*I*) was calculated using the equation: $I = -\Sigma pi\ log2\ pi$; and genetic diversity (*h*) was calculated using the equation: $h = 1 - \Sigma pi2$ (Peakall & Smouse, 2012). Haplotypic frequencies were calculated and a median-joining network analysis performed using the NETWORK 5.0.0.1 software (Fluxus Technology Ltd., Suffolk, England).

Morphological and agronomic characterization of cowpea landraces

Phenotypic characterization was performed in 50 *V. unguiculata* subsp. *unguiculata* cultigroup unguiculata landraces from Southern Europe, focusing in Iberian Peninsula landraces (25 Portuguese and 17 Spanish), 4 Italian and 4 Greek (Supplementary Table S1). One trial was installed at the University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal (N 41°17′51″, W 07°44′12″, 465 m.a.s.l.). Sowing was carried out in the first week of June 2016. From each landrace, 10 seeds were hand sown in one row 2 m in length, with a distance of 0.75 m between rows and 0.20 m between seeds. The topsoil (0–20 cm) was classified as gleyic fluvisol with a medium texture and 1.61 g/kg humus content, 44 mg/kg of P_2O_5, 110 mg/kg of K_2O_2 and a pH (KCl) 5.2. Before sowing, the experimental field was ploughed with a rotary tiller and fertilized with 250 kg/ha of nitro-magnesium 27 and 200 kg/ha of NPK (Ca-Mg-S) 8-12-12 (2-2-14). The average solar radiation, air temperature, precipitation, and relative humidity per month (from May to September) were recorded at weather stations located in the experiment location (see Supplementary Table S2). The set of 50 landraces were phenotyped using six qualitative characters (growth habit, terminal leaflet shape, flower colour, seed coat colour, hilum colour and seed shape) and four quantitative characters (pod length, number of seeds per pod, 100 seed weight and total seed weight) based on the descriptors for cowpea proposed by the International Board for Plant Genetic Resources (IBPGR) (IBPGR, 1982). For the quantitative characters of pod length and number of seeds per pod, five random pods were analysed and for the parameter 100 seed weight, two random samples of the total seed produced by each accession were weighed.

Multivariate analysis was carried out on the phenotypic data (see Supplementary Table S3), through principal component analysis (PCA) and the construction of the dendrogram by the Ward method, using the Past3 program (Hammer et al., 2001). A total of 50 accessions × 10 plants were used to agro-morphological characterization. The qualitative traits frequencies were determined manually. Minimum, maximum and mean values, standard deviations, coefficients of variation, F value and heritability were calculated for the quantitative traits. The heritability of each quantitative trait was calculated using the following equation: $h^2 = (s_g^2)/[s_g^2 + (s_e^2/r)]$, where s_g^2 and s_e^2 represent the genetic and residual variance for each trait, and r the number of replicates of each landrace (Gitonga et al., 2014). The treatment of quantitative data and the calculation of significant differences with the Tukey test were performed using the summary statistics procedure in the SPSS program version 8.0.

Results

Chloroplast SSRs polymorphism

A total of 10 pairs of primers were used to analyse the genetic diversity of 113 *Vigna* accessions. Eight (ccmp3, ccmp7, ccSSR4, cSSR7, VgcpSSR1, VgcpSSR10, VgcpSSR12 and VgcpSSR14) out of the ten chloroplast microsatellite loci screened were polymorphic (see Supplementary Table S4). The number

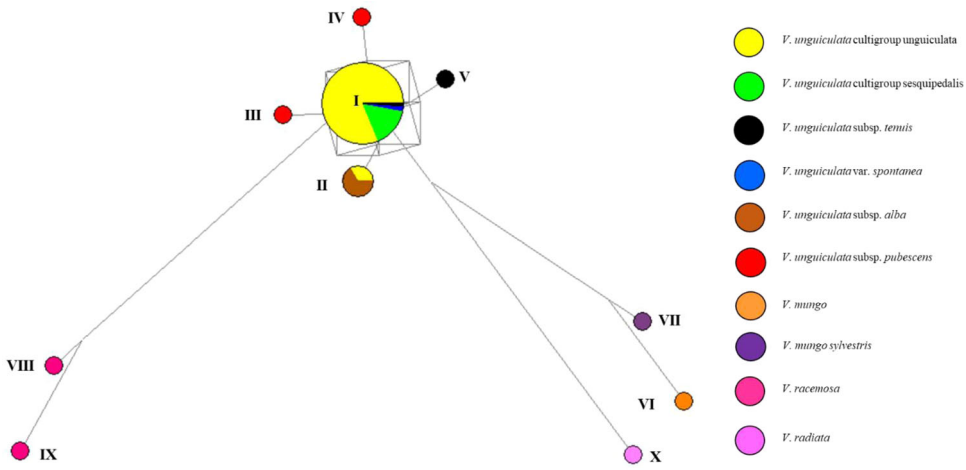

Fig. 1. Median-joining network of the haplotypes observed in 113 *Vigna* accessions (the area of the circles is proportional to haplotype frequency).

of amplified alleles per primer pair ranged from one to five (Table 2). Genetic diversity varied from 0.000 to 0.135 and was measured by the allele variation at the ten loci, with the minimum value in the polymorphic loci of 0.035 in the ccmp3 locus, and the maximum value of 0.135 in the locus VgcpSSR14 (Supplementary Table S4). The number of effective alleles (*Ne*) and Shannon's information index (*I*) was also higher in the locus VgcpSSR14 (Supplementary Table S4).

Relationships between *Vigna* germplasm

In the Median-joining network (Fig. 1), it is possible to observe the ten different cpSSR haplotypes found, being eight of them unique (taxon specific). The most frequent haplotype (I) included the cultivated *V. unguiculata* subsp. *unguiculata* cultigroups unguiculata and sesquipedalis, *V. unguiculata* subsp. *unguiculata* var. *spontanea* and the specimen of *V. unguiculata* subsp. *tenuis* from Mozambique. The second most frequent haplotype (II) included the *V. unguiculata* subsp. *unguiculata* from the Democratic Republic of Congo and specimens of *V. unguiculata* subsp. *alba*. Unique haplotypes (III-X) were established for the taxa *V. unguiculata* subsp. *pubescens* (haplotypes III and IV), *V. unguiculata* subsp. *tenuis* (from Zambia) (haplotype V), *V. mungo* and *V. mungo sylvestris* (haplotypes VI and VII, respectively), *V. racemosa* (haplotypes VIII and IX) and *V. radiata* (haplotype X).

Morphological and agronomic diversity of cowpea landraces

During the period of characterization, the months of July and August registered the highest mean temperatures, as in the long-term period from 1981–2010 (Supplementary Table S2). However, the maximum temperature absolute values were much higher in 2016. In terms of precipitation, 2016 was significantly different compared to the long-term period 1981–2010, with the most pronounced differences being high precipitation in May and the very dry months of July and August with precipitation not exceeding 0.2 mm (Supplementary Table S2).

This study verified a high variability for the traits analysed in the 50 landraces from Southern Europe (see Fig. 2 and Supplementary Tables S3 and S5). Different types of growth habits were verified in this set of landraces, with the erect growth habit being the most frequent (46%). The prostrate growth habit was found exclusively in Greek landraces. Sub-hastate were the most common terminal leaflet type shapes (42%). Two different flower colours, white (78%) or purple (22%), were observed. The most common seeds were cream-coloured (94%), black hilum (58%) and kidney-shaped (66%). In addition, the cream seeds were mostly from the Iberian Peninsula and black and brown seeds were only observed in the Italian and Greek landraces, respectively, and grey hilum in landraces from Spain (see Supplementary Table S5).

For the four quantitative traits: pod length, number of seeds per pod, 100 seed weight and total seed weight, the minimum, maximum and mean values, the standard deviation, coefficient of variation, F value and heritability were determined (Table 3). Pod length ranged from 11.4 cm (Vg208) to 21.1 cm (Vg187); the seed number per pod ranged from 7 (Vg95) to 14 (Vg59); one hundred seed weight from 12.1 g (Vg91) to 36.9 g (Vg191) and the total seed weight per plant from 5.32 g (Vg162) to 95.50 g (Vg97). The

Fig. 2. *Vigna unguiculata* subsp. *unguiculata* (L.) Walp cv. gr. *unguiculata* morphological diversity.

parameter that presented the highest F value was the 100 seed weight (103.05). Total seed weight per plant was the character with the highest coefficient of variation (78.48%) and pod length the one with the lowest (14.47%) (Table 3). When the heritability was classified into high (>0.75), moderate (0.60–0.75) and low (<0.60), the parameter 100 seed weight presented a high heritability ($h^2 = 0.98$), pod length a moderate heritability ($h^2 = 0.66$) and the number of seeds per pod a low heritability ($h^2 = 0.28$) (Table 3).

The principal component analysis (PCA) showed that the first three principal components explain 71.4% of the total variation (PC1 = 35.9%; PC2 = 22.4% and PC3 = 13.1%) (Fig. 3 and Table 4). The traits that contributed most to the separation of the landraces were: in the first component, the hilum colour (HC) (0.51); in the second component, the total seed weight per plant (TSW) (0.79) and in the third component, the terminal leaflet shape (TLS) (0.88) (Table 4). The PCA enabled the discrimination of cowpea landraces into three main groups, all of which include Portuguese landraces showing the diversity present on these landraces and the potential of them in future breeding programs. The largest group, designated I, and distributed mainly in the second and third quadrants, comprises Portuguese, Spanish and Italian landraces; a second group (II) positioned in the first quadrant, comprises six landraces (the Portuguese Vg212 and Vg245, the Spanish Vg62 and Vg97, the Italian Vg206 and the Greek Vg208), and a third group (III) also comprising six landraces, namely, two from Portugal (Vg52 and Vg91), one from Italy (Vg204) and three from Greece (Vg161, Vg162 and Vg209), positioned in the fourth quadrant.

Discussion

In this study, morphological, agronomic, and molecular characterization was carried out in 50 landraces of *Vigna unguiculata* subsp. *unguiculata*. The 10 pairs

Table 3. Mean values obtained for each of 50 cowpea landraces for each quantitative trait with their respective mean, standard deviation (SD), coefficient of variation (CV), heritability (h^2), F value and Tukey's test (for a significance level of 0.05).

Accession number[a]	Pod length (cm)	Number of seeds per pod	100 seed weight (g)	Total seed weight per plant (g)
Vg11	18.5	9.6	26.8	10.91
Vg12	18.7	10.6	23.4	11.65
Vg13	18.6	10.6	21.0	9.40
Vg15	20.4	12.0	25.5	11.67
Vg18	20.6	12.0	26.4	20.30
Vg47	18.7	10.6	28.3	10.25
Vg49	17.3	9.0	22.7	5.82
Vg51	19.5	10.8	25.4	14.23
Vg52	18.2	12.0	22.4	11.97
Vg54	20.3	11.6	26.3	31.63
Vg56	17.9	12.2	22.8	15.95
Vg58	17.8	12.4	19.2	14.86
Vg59	18.0	14.0	15.0	21.03
Vg60	16.7	12.0	19.3	31.94
Vg62	14.6	12.2	13.8	37.07
Vg64	20.5	12.6	22.9	30.39
Vg72	19.0	10.2	24.2	8.93
Vg91	14.5	10.4	12.1	5.83
Vg94	19.8	13.2	22.0	13.33
Vg95	16.4	7.0	32.4	12.60
Vg97	17.5	10.8	19.5	95.50
Vg99	20.1	12.6	21.3	13.55
Vg101	17.1	8.0	26.8	7.10
Vg104	19.9	10.8	19.8	33.30
Vg161	15.8	12.2	17.1	21.83
Vg162	12.5	10.2	14.1	5.32
Vg187	21.1	11.6	25.0	19.50
Vg191	19.8	10.4	36.9	26.11
Vg193	16.2	10.4	16.5	21.10
Vg204	16.0	9.8	19.5	22.08
Vg206	15.0	9.8	20.3	48.07
Vg208	11.4	10.2	16.9	47.83
Vg209	13.5	11.0	16.5	13.00
Vg212	18.6	11.0	22.9	58.00
Vg217	16.8	11.0	24.5	27.33
Vg220	20.4	10.4	26.3	26.20
Vg222	21.0	10.4	27.2	18.30
Vg223	18.3	13.0	24.1	23.25
Vg224	15.1	11.0	16.8	12.90
Vg232	17.8	10.6	24.3	7.40
Vg236	17.4	11.2	22.1	12.17
Vg239	19.2	12.0	23.2	15.29
Vg241	17.6	9.8	24.8	7.16
Vg243	15.5	12.6	15.6	20.00
Vg244	17.4	10.2	24.2	7.96
Vg245	15.9	12.8	23.3	59.93
Vg248	16.7	10.4	24.4	11.69
Vg249	19.0	11.4	29.0	11.30
Vg251	17.4	12.0	20.7	26.50
Vg252	20.6	12.6	27.9	15.76
Average	17.73	11.09	22.44	21.30
SD	2.57	1.97	4.84	16.72
CV (%)	14.47	17.73	21.54	78.48
h^2	0.66	0.28	0.98	–
F	11.14	3.06	103.05	–
$Tukey_{0.05}$	2.55	2.84	1.15	–

[a]Details on Table 2.

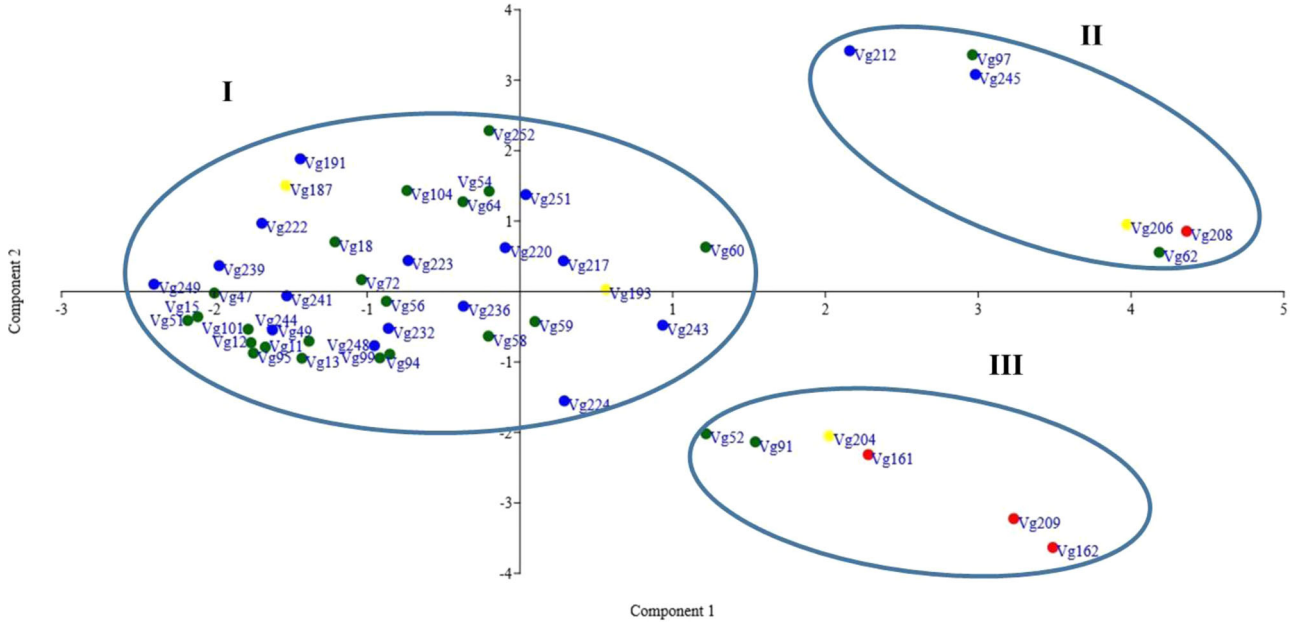

Fig. 3. Principal Component Analysis (PCA) of 50 cowpea landraces based on 10 agronomic traits (green – Portuguese landraces; blue – Spanish landraces; yellow – Italian landraces; red – Greek landraces).

Table 4. Association of coefficients and vectors with the three axes of principal component analysis.

	PC1	PC2	PC3
Eigenvalues	3.5	2.2	1.3
Percentage	35.9	22.4	13.1
Cumulative percentage	35.9	58.3	71.4
	PC1	**PC2**	**PC3**
PL	–0.41	0.32	0.02
NSP	0.06	0.05	0.26
SW	–0.33	0.22	–0.25
GH	0.35	0.03	–0.11
TLS	0.05	0.24	0.88
FC	0.09	–0.06	–0.01
TSW	0.50	0.79	–0.23
SC	0.05	–0.07	0.03
HC	0.51	–0.39	–0.05
SS	0.25	–0.03	0.18

(PL, Pod Length; NSP, Number of Seeds per Pod; SW, 100 Seeds Weight; GH, Growth Habit; TLS, Terminal Leaflet Shape; FC, Flower Colour; TSW, Total Seed Weight per plant; SC, Seed Colour; HC, Hilum Colour; SS, Seed Shape).

of primers of cpSSRs allowed to verify a low level of polymorphism within the cultivated cowpeas, which is in agreement with previous studies reported for cowpea (Pan et al., 2014; Shukla et al., 2018) and other crops such as the common bean (*Phaseolus vulgaris*) (Desiderio et al., 2013).

Moreover, the same set of 10 cpSSRs were used to evaluate genetic diversity and phylogenetic relationships among 113 *Vigna* accessions including *Vigna unguiculata* subsp. *unguiculata*, subsp. *alba,* subsp. *pubescens,* subsp. *tenuis* and var. *spontanea* and, accessions from other *Vigna* species as *V. mungo*, *V. radiata* and *V. racemosa*. A total of ten different cpSSRs haplotypes were identified in this study. The haplotypes network (Fig. 1) suggests that haplotype I may correspond to an ancestral type as it is the most abundant and widespread haplotype and from this haplotype diverge the remaining ones. It is also possible to confirm, as expected, that the species *V. radiata*, *V. mungo* and *V. racemosa* are more distant from the cowpea ancestor *V. unguiculata* subsp. *unguiculata* var. *spontanea* than the different subspecies of *V. unguiculata*.

In this study it was not possible to differentiate the unguiculata and sesquipedalis cultigroups, and these two cultigroups shared a haplotype with the wild var. *spontanea*. Results are in line with other studies, where it was not possible to distinguish both cultigroups using cpSSR markers. Fatokun et al. (1993) and Vijaykumar et al. (2012) have already verified that these cultigroups are closely related, also with its wild relative var. *spontanea*. It is known that both cultivated cowpea forms are products of a post-domestication evolution of *V. unguiculata* in different parts of the world (Fatokun et al., 1993). Whereas the African use of the unguiculata cultigroup remained unchanged over time, the sesquipedalis cultigroup became established as a long-podded vegetable in Asia (Smartt, 1985). The shared haplotype between these two forms of cultivated cowpea suggests

a highly conserved nature of the chloroplast genome with a low mutation rate in the worldwide germplasm of cultivated forms of *V. unguiculata* subsp. *unguiculata* (Kapil et al., 2014; Palmer, 1985; Vaillancourt & Weeden, 1992). Nuclear SNP markers enable population structuring of cultivated cowpea that differentiates between sesquipedalis and unguiculata germplasm, regardless of the origin of the sesquipedalis material (Carvalho, Muñoz-Amatriaín, et al., 2017).

When analysing all the accessions under study, the accession belonging to *V. unguiculata* cultigroup unguiculata from the Democratic Republic of Congo revealed the same cpSSR haplotype as the subsp. *alba,* rather than the haplotype of its ancestor (var. *spontanea*). This can be explained by the same geographic origin of the different taxa. Also, in a PCA analysis based on nSSR markers, Lioi et al. (2019) reported the positioning of Congo germplasm in a quadrant different from that of the Italian germplasm from the southern region of Apulia, and Carvalho, Muñoz-Amatriaín, et al. (2017) reported with SNP markers a population structure of worldwide cowpeas with south European germplasm clustering with North African but not with Southeast African germplasm.

Accessions of the subsp. *tenuis* and subsp. *pubescens* showed two different haplotypes. In the case of subsp. *pubescens,* neither of the two haplotypes was equal to the var. *spontanea* haplotype. Pasquet (1998) with isozyme diversity analysis in several *V. unguiculata* subspecies divided the subsp. *tenuis* into an inland outcrossing cluster and a coastal cluster. Moreover, the same author mentions divergences at accessions level between var. *spontanea* reflecting introgressive hybridizations, namely with subsp. *pubescens*. Nevertheless, further molecular studies would be needed, namely increasing the number of loci to genotype and sampling of cultivated cowpea in different locations, to better understand the genetic basis of this crop phylogenetic relationships analysing wild relatives, including other species of *Vigna* and subspecies of *V. unguiculata.*

The morphological and agronomic characterization allowed the evaluation of the performance of 50 landraces from Southern Europe. Knowledge of phenotypic variation and genotype relationships is essential to help plant breeders develop appropriate breeding strategies and create the most adaptive and productive cultivars. The characterization performed in this study allowed to verify the high diversity among Southern European cowpea landraces, which may be useful in future breeding programs to obtain new varieties. Qualitative traits are generally independent of environmental factors and are governed by one or few major genes (Govindaraj et al., 2015) quantitative traits are influenced by environmental factors. As the grain-type cowpea is the most widely consumed in Southern Europe, the morphological characterization was carried out for one set of 50 landraces of the cultigroup unguiculata, mainly from the Iberian Peninsula but also from Italy and Greece. Considering the yield trait 'total seed weight per plant', the five accessions with the greatest total seed weight results [47.8–95.5 g] comprise landraces from the four Southern countries: a Portuguese landrace (Vg97 from the Abrantes region in the centre of Portugal), followed by two Spanish landraces (one from the northern province of Galicia and another from Eastern Castilla La Mancha), one from Italy (Sicily) and the last one from Greece. In this study the 100 seed weight parameter presented a high heritability ($h^2 = 0.98$), pod length a moderate heritability ($h^2 = 0.66$), and the number of seeds per pod a low heritability ($h^2 = 0.28$) (Table 3). Omoigu et al. (2006) and Egbadzor et al. (2013) also reported in cowpea a high heritability in the 100 seed weight parameter, $h^2 = 0.98$ and $h^2 = 0.96$, respectively. High heritability for 100 seed weight ($h^2 = 0.91$) was also verified in soybean (*Glycine max* (L) Merrill) in a study by Aditya et al. (2011). However, to perform efficient selection, high heritability should be accompanied by high genetic advance (i.e., improvements in the value of selected plants over the parental population) indicating that selection should lead to fast genetic improvement of the material (Eid, 2009; Mishra et al., 2014).

Several studies on cowpea report that pod length has a moderate to high heritability, as in the current study. Apte et al. (1987) verified a heritability of $h^2 = 0.62$, Patil and Baviskar (1987) observed a heritability of $h^2 = 0.70$ and Thiyagarajan (1989) showed a heritability of $h^2 = 0.71$ in this characteristic. In the case of seeds per pod, some authors also verified a low heritability, such as Patil and Baviskar (1987) who verified a heritability of 0.33 and Sreekumar et al. (1979) of 0.41. In this characterization, no evident relationship was observed between the geographic origin of the 50 landraces and their clustering based on the morphological and agronomic traits analysed. This may reflect trade in cowpea material within Southern European countries, particularly the Iberian Peninsula, and also a common origin of the cowpea germplasm cultivated in this part of Europe, which is corroborated by the good agro-morphological performance of Italian and Greek genotypes in a Portuguese environment field experiment.

The morphological and agronomic characterization of cowpea landraces showed great diversity among Southern European cowpea landraces, which will be useful in future breeding programs to obtain new varieties for Southern European countries by providing

agro-morphological information about its germplasm, with a focus on those from the Iberian Peninsula.

As cowpea is a legume that is resistant to different biotic and abiotic factors and contains important nutritional factors, its characterization, both morphologically and molecularly, is extremely important for a precise taxonomy, an understanding of phylogenetic relationships, and for introducing local germplasm into breeding programs.

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Supplemental data

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ORCID

Isaura Castro http://orcid.org/0000-0003-3082-4214
Márcia Carvalho http://orcid.org/0000-0003-1108-8389
Juan Pedro Martín http://orcid.org/0000-0002-8180-5489
Eduardo Rosa http://orcid.org/0000-0001-5296-3843
Valdemar Carnide http://orcid.org/0000-0001-9769-9636

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