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Analysis of variability and phylogeny in pisum (*Pisum spp.*) using digital phenotyping and morphological traits

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Abstract

Plant phenotyping links genomics with plant ecophysiology and agronomy. It is usually performed by non-destructive, automated and image-based technology and generates information for efficient and searchable digital characterization of crop that can be performed during routine, periodical regeneration of accessions in germplasm collections. In the present work, ninety-two accessions of *Pisum* from different species and subspecies were studied during 2015 and 2016. Size and colour traits were measured using digital images from a Samsung CLX 3300 scanner and analysed with appropriate software; also seed weight, plant height and days to flowering were measured. Highly significant differences between accessions and species and subspecies for all these traits were found. When distances among species and subspecies are calculated, *P. sativum* subsp. *sativum* showed the greatest distance with *P. fulvum* (8.02) followed by *P. abyssinicum* (7.13); while the smallest distance was found between *P. fulvum* and *P. sativum* subsp. *transcaucasicum* (3.16). A Neighbour-joining tree with a cofenetic r of 0.985 was obtained. Seed and pod characteristics as colour parameters and size, obtained by digital phenotyping, have proved to be suitable markers for genetic diversity evaluation and they are useful in evolutionary analysis, allowing the discrimination of the main wild and cultivated species in the genus *Pisum.*

Keywords: Pisum, seed coat colour, origin, variability, phenotyping, legumes.
Abbreviations: a_and b- colour coordinates from the CieLab system of colour, C_ calibre of grains in centimetres, CR_ coincidence rate, CV%_ variation coefficient, DF_ days until 50% of plants flowering, L_ psychometric index of lightness from the CieLab system of colour; Max_ maximum , MD%_ mean difference percentage , Min_ minimum, PD_ pod diameter in centimetres, PH_ plant height in centimetres, PL_ pod length in centimetres, RBIP_ retrotransposon-based insertion polymorphisms markers, RGB_ red, green and blue, SD_ standard deviation , SSAP_ sequence-specific amplification polymorphism markers, VD%_ variance difference percentage, VR%_ variable rate of coefficient of variance, W100S_ weight of 100 seeds in grams.

Introduction

Pea (*Pisum sativum* L.) is one of the world's oldest domesticated crops. It is the third most widely grown legume, as its seeds serve as a protein-rich food for humans and livestock alike. Domesticated about 10,000 years ago (Zohary and Hopf, 2000), pea is currently cultivated in temperate zones worldwide. Genus is thought to have originated in northwest Asia, where most subspecies were found.

Several studies have been performed to analyze genetic diversity among wild and domesticated forms of *Pisum* using molecular methods and to infer phylogeny, evolution and domestication. Some examples of these are the investigations performed by Vershinin et al. (2003), who studied the genetic structure and evolutionary history of the genus using Sequence-Specific Amplification Polymorphism (SSAP) markers; Ellis et al. (2005) concluded that the exceptional DNA polymorphism within this genus is associated with recent genome expansion; Jing et al. (2007) analysed sequence diversity of 39 dispersed gene loci and concluded that the different genes show large variation in diversity parameters, suggesting widely differing levels of selection and a high overall diversity level for the species. Martin-Sanz et al. (2011) analysed accessions of different wild and cultivated *Pisum* sp. using retrotransposon-based insertion polymorphisms (RBIP) markers and found that landraces maintain a relatively high variability which is only partially represented in cultivars generally sown in Spain and are still a source of genetic variability for breeding new pea cultivars. Smýkal et al. (2011) studied 4429 *Pisum* accessions from three large world germplasm collections that include both wild and domesticated pea and found that introgression from wild species has been common and much of the diversity still resides in wild material and could be used further in breeding.
Smýkal et al (2014), analysing ancient DNA (aDNA) extracted from carbonized pea seeds recovered from deposits at Hissar, in southeast Serbia, that date to the eleventh century B.C. placed

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the ancient sample at an intermediate position between extant cultivated *Pisum sativum* L. and wild *P. sativum* subsp. *elatius* (Steven ex M. Bieb.) Asch. et Graebn. and concluded that the material represents an early domesticated pea, possibly of winter type with coloured flower and pigmented testa, similar to today's fodder pea (*P. sativum* subsp. *sativum* var. *arvense* (L.) Poir.).

According to Patwardhan et al. (2014), biomolecular markers have become favourable because there are many more molecular characters available, their interpretation is generally easier, there is no sampling bias involved, are easier to obtain and are subject to robust statistical analysis methods. In molecular phylogeny, the relationships among organisms or genes are studied by comparing homologues of DNA or protein sequences, where dissimilarities indicate genetic divergence as a result of molecular evolution during the course of time. The concept is based on a steady rate of change in DNA sequences over time and provided a basis for dating the time of divergence of lineages, but this hypothesis has been questioned many times because biomolecules are subjected to changes at different rates.

Domestication of many seed crops (Weeden 2007) has involved similar modifications in a reduced set of traits including seed dispersal, seed dormancy, gigantism and increased harvest index, and relatively few genes appear to have been modified during the domestication of pea.

Abbo et al. (2014) propose that only traits showing a clear domesticated–wild dimorphism represent the pristine domestication episode, whereas traits with a clear phenotypic continuum between wild and domesticated gene pools cannot be used to discuss or to describe plant domestication and cannot provide support for a protracted domestication model because mostly reflect post-domestication diversification. In pea, the traits crucial in domestication are shattering, dormancy mediated by water-impermeable coats, seed size and vernalisation. Gepts (2004) proposed that seed size can generally be used to study pea domestication and seed colour and pod shape may be additional possibilities.

Plant phenotyping is an emerging science that links genomics with plant ecophysiology and agronomy. It is usually performed during routine, periodical regeneration of accessions by non-destructive, automated and image-based technology and generates an enormous amount of information for efficient and searchable digital characterization of crops. Visual assessments made using colour, size, shape and texture are simple, but they can be highly subjective, so, digital image analysis is a methodology to overcome this problem because it offers an objective and quantitative method for estimation of morphological parameters.

Dell' Aquila (2009) stated that the application of different image analysis system prototypes in monitoring seed germination of several species has provided encouraging results along with the possibility to determine the colour space of a two-dimensional seed surface. Working on lentil seed germination, he concluded that quantitative changes in Red-Green-Blue (RGB) colour component density may be considered as markers of the start of germination and can be used in classifying sub-samples and maintaining high germination quality in aged seed samples represents a non-destructive method in seed testing and sorting. Digital image analysis is a non-destructive technique increasingly utilized in phenotyping various parameters of plant health status such as chlorophyll content (Dutta Gupta et al. 2013), nitrogen content (Vollmann et al. 2011), and disease status (Porob et al. 2017).
Seed banks contain enormous amounts of germplasm, which is only partially characterized. While significant progress has been made in molecular and genetic approaches in recent years, the quantitative analysis of plant phenotypes-structure and function of plant - has become the major bottleneck.

The aim of this investigation in to analyse the variability present in a work collection of *Pisum* through plant's morphological characters an digital phenotyping of seeds and pods and investigate if these traits used are suitable to infer phylogenetic relations among the species and subspecies used.

Results and discussion

Genetic variation

Mean values, Standard Deviation (SD), Minimum (Min) and Maximum (Max) values, Variation coefficient (CV%) and F value from the ANOVA between accessions were calculated (Table 2). The Fs values show that there are highly significant differences between accessions for all these traits. Hue presented the major variability, while DF showed the lowest one.

Assessing crop genetic variation is vital to understanding the available genetic variability and potential use for varietal improvement. Phenotyping has been applied to different crops collections. Congling et al. (2015) used phenotyping of peanut pod and seed traits for cultivar selection and genetic mapping of grade components in a group of peanut germplasm with high levels of phenotypic variation. Fedoruk et al. (2013) phenotyped a recombinant inbred line population for seed shape, colour, and pattern of lentil (*Lens culinaris* Medik. subsp. *culinaris*) over multiple site–years and classified them according to cotyledon and seed coat colour and pattern, which are important characteristics in determining the market class and the end uses of the crop.

One of the traits modified during domestication of plants is the dormancy of the seeds. The capacity to germinate in pea is determined by the capacity of water imbibition of the seed, related to the permeability of its coats. Troszyńska et al. (2002) stablished that condensed tannins occurring in the coloured seed are located mainly in the seed coats (hulls) and play an important role in the defines system of seeds that are exposed to oxidative damage by many environmental factors such as light, oxygen, free radicals and metal ions. Xiaofang et al. (1998) found that environment was less significant than the genotype in the determination of total phenolic levels in pea, and seeds with darker seed coat colour contained higher levels of condensed tannins. Also, Smulikowska et al (2001) pointed that the digestible energy value is similar to soybean (*Glycine max* L. Merr.) meal and the metabolizable energy values are higher for grains for white flowered peas and is slightly lower for purple flowered peas (flower colour is a trait associated to pigmented seeds) According to Stanek et al (2004) pigmented seeds have less crude protein and Bastianelli et al (1998) pointed that monogastric animals digest less effectively the seeds rich in tannins. Thus the preference of seeds with no pigmented teguments may have been conditioned also during the domestication of *Pisum* by these facts.
When an ANOVA between species and subspecies is performed (Table 3), the a coordinate of colour didn't showed differences;

Table 1: Common name, classification and source of accessions.

P. fulvum		*P. sativum*	
P. fulvum	JI 224*	*P. sativum ssp transcaucasicum*	
JI 1006	JI 1006	B1448	USDA 639982
		Virk2376	USDA 639971
P. elatius		*P. sativum ssp jomardii*	
Vir2524E27	USDA 15008+	JIC 250	JIC 250
Vir3115	USDA 639973	*P sativum ssp sativum*	
Vir2521	USDA 15007	Afila	JI 3509
CE-1	JI 2629	Arvejas Amarillas	USDA 109865
		Ervilha T Flor R	USDA 117998
P. abyssinicum		NN16	commercial cultivar
B54757	USDA 15041	Patani colour	USDA 123246
B808	USDA 639983	Zavalla16	breeding line
		Zavalla23	breeding line

*JI: John Innes Centre, Norwich, UK. http://www.jic.ac.uk/GERMPLAS/pisum, +USDA: Plant Germplasm Introduction and Testing, USA. http://www.ars-grin.gov

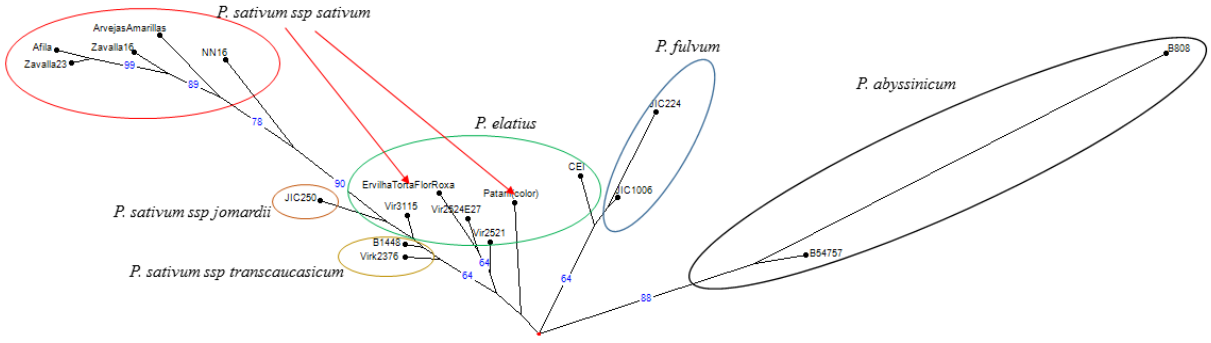

Fig 1. Neighbor-joining tree for different *Pisum* species and subspecies. Bootstrap values (10,000 replicates) superior to 60 are indicated on each node of the tree. *P. sativum* subsp. *sativum*, *P. elatius*, *P. fulvum*, *P. abyssinicum*, *P. sativum* subsp. *jomardi*i and *P. sativum* subsp *transcaucasicum*.

Table 2. Mean values, Standard Deviation, Minimum and Maximum values, Variation coefficient and F value for the evaluated traits.

Traits	Mean ± SD	Min	Max	CV%	F value
W100S (g)	12.80 ± 7.69	3	27	60.06	17.04***
C (cm)	0.59 ± 0.16	0.34	0.88	27.17	69.44***
DF	102.26 ± 16.87	68	143	16.49	4.42***
PH (cm)	119.22 ± 46.53	36.5	199	39.03	21.34***
PL (cm)	5.72 ± 1.70	3.07	8.80	29.71	19.89***
PD (cm)	1.08 ± 0.30	0.58	1.73	27.48	3.88***
Hue	70.87 ± 63.01	20.56	309.94	88.90	96.23***
Chroma	19.49 ± 6.50	5.56	33.82	33.37	13.72***
a	12.07 ± 6.22	1.42	24.53	51.53	13.87***
b	13.75 ± 6.65	-4.75	24.53	48.38	40.58***
L	29.68 ± 16.54	4.36	58.27	55.71	124.46***
Red	97.56 ± 41.70	16.99	169.75	42.74	220.71***
Green	62.57 ± 40.68	10.63	134.08	65.01	84.35***
Blue	53.30 ± 35.03	10.23	118.96	65.71	73.04***

*** $p < 0.001$. Days until 50% of plants flowering (DF); weight of 100 seeds (W100S); plant height (PH); calibre of grains (C); pod length (PL); pod diameter (PD); colour coordinates (a and b) and psychometric index of lightness (L) from the CieLab system of colour; parameters Red, Green and Blue, Hue angle value and Chroma index from the RGB system of colour.

Table 3. Mean values with the corresponding standard error and F value from ANOVA between species and subspecies.

Traits	*P. abyssynicum*	*P. fulvum*	*P. elatius*	*P. sativum*			F value
				subsp. *sativum*	subsp. *jomardii*	subsp. *transcaucasicum*	
W100S (g)	11.2 ± 1.9^{b}	3.5 ± 2.3^{c}	10.4 ± 1.6^{bc}	20.4 ±1.2^{a}	10.5 ± 3.2^{bc}	4.0 ± 2.3^{c}	14.7***
C (cm)	0.5 ± 0.04^{bc}	0.4 ± 0.05^{cd}	0.5 ± 0.03^{b}	0.7 ± 0.03^{a}	0.5 ± 0.07^{bcd}	0.4 ± 0.05^{d}	13.5***
DF	104.2 ± 6.1^{ab}	88.2 ± 7.5^{b}	105.9 ± 5.3^{ab}	95.9 ± 4.0^{b}	122.0 ± 10.6^{a}	118.5 ± 7.5^{a}	2.9**
PH (cm)	137.8 ± 17.2^{a}	54.5 ± 21.1^{b}	126.2 ± 14.9^{a}	117.2 ± 11.3^{a}	160.7 ± 29.8^{a}	128.5 ± 21.1^{a}	2.6*
PL (cm)	5.8 ± 0.4^{a}	3.2 ± 0.6^{c}	5.8 ± 0.4^{a}	6.9 ± 0.3^{a}	5.5 ± 0.8^{ab}	3.7 ± 0.6^{bc}	9.3***
PD (cm)	1.10 ± 0.1^{a}	0.8 ± 0.1^{bc}	1.0 ± 0.1^{ab}	1.3 ± 0.1^{a}	1.0 ± 0.2^{abc}	0.7 ± 0.1^{c}	6.7***
Hue	166.9 ± 19.9^{a}	27.4 ± 24.4^{b}	48.6 ± 17.2^{b}	61.9 ± 13.0^{b}	73.5 ± 34.5^{b}	44.7 ± 24.4^{b}	5.9***
Chroma	13.8 ± 2.4^{b}	14.4 ± 3.0^{ab}	21.4 ± 2.1^{a}	20.6 ± 1.6^{a}	23.9 ± 4.2^{a}	23.2 ± 3.0^{a}	2.5*
a	10.6 ± 2.5	12.2 ± 3.1	14.1 ± 2.2	11.0 ± 1.7	6.7 ± 4.4	16.5 ± 3.1	1.0^{ns}
b	4.5 ± 1.8^{b}	7.0 ± 2.2^{b}	15.7 ± 1.6^{a}	16.6 ± 1.2^{a}	22.4 ± 3.1^{a}	16.2 ± 2.2^{a}	10.2***
L	11.6 + 4.7^{d}	13.9 + 5.8^{cd}	25.3 ± 4.0^{bc}	43.4 ± 3.1^{a}	36.8 ± 8.2^{ab}	29.7 ± 5.8^{bc}	8.8***
Red	47.2 + 11.2^{d}	55.8 + 13.7^{cd}	89.0 ± 9.7^{bc}	132.3 ± 7.3^{a}	111.0 ± 19.4^{ab}	103.4 ± 13.7^{ab}	10.6***
Green	22.6 + 12.3^{c}	27.0 + 15.1^{c}	50.0 ± 10.7^{bc}	95.6 ± 8.1^{a}	80.9 ± 21.4^{ab}	58.2 ± 15.1^{bc}	7.0***
Blue	25.6 + 11.4^{b}	27.3 + 13.9^{b}	39.1 ± 9.9^{b}	81.9 ± 7.5^{a}	54.2 ± 19.7^{ab}	48.7 ± 13.9^{b}	5.4***

Means with same letter don't differ significally in the LSD test. ^{ns} not significant; * significant p<0.05; **significant p<0.01 and *** p significant p<0.001. Days until 50% of plants flowering (DF); weight of 100 seeds (W100S); plant height (PH); calibre of grains (C); pod length (PL); pod diameter (PD); colour coordinates (a and b) and psychometric index of lightness (L) from the CieLab system of colour; parameters Red, Green and Blue, Hue angle value and Chroma index from the RGB system of colour.

Table 4. Euclidean distances (standardized data) among species and subspecies

	P. abyssinicum	*P. fulvum*	*P. elatius*	*P. sativum* subsp. *sativum*	*P. sativum* subsp. *jomardi*
P. fulvum	4.83				
P. elatius	4.24	4.61			
P. sativum subsp. *sativum*	7.13	8.02	4.65		
P. sativum subsp. *jomardi*	6.06	6.99	3.55	4.50	
P. sativum subsp. *transcaucasicum*	6.05	5.05	3.16	6.48	4.25

there were significant differences for Chroma ($F=2.5$ $p<0.05$), PH ($F=2.6$ $p<0.05$) and DF ($F=2.9$ $p<0.01$) while the rest of the variables analysed showed highly significant differences with $p<0.001$.

P. sativum subsp. *sativum* presented the highest W100S and C. *P. fulvum* showed the lowest PH. *P. abyssynicum* presented the highest Hue value. Regarding the b coordinate of colour, these two species showed lower values with no significant differences among them, while *P. elatius* and all subspecies of *P. sativum* showed higher ones.

P. abyssynicum and *P. fulvum* had darker seed because presented least L values (L=0 is black, L=100 is white) while *P. sativum* subsp. *sativum* and subsp. *jomardii* had seeds with higher L values. This difference can be explained by the fact that most of accessions of subsp. *sativum* used in human alimentation have very thin teguments that have different grades of transparency due to different degrees of white colouration, while other accessions have solid pigmented teguments, with no transparency at all. The "b" coordinate showed that *P. abyssynicum* and *P. fulvum* had similar blueness in opposite to the rest of the species and subspecies, with major yellowness in seed colour.

P. abyssynicum had the highest Hue value, indicating a colour more proximal to pure cyan than green; *P. fulvum* had the lowest value, with no differences with *P. elatius* and *P. sativum* subsp. *transcaucasicum*, indicating a colour proximal to red; while *P. sativum* subsp. *sativum* and subsp. *jomardii* showed colour between yellow and green. These differences in Hue indicate variability in the presence and concentration of tannins and other phenolic compound in the tegument of the seeds correlated to impermeability, but farther analysis is needed to elucidate if the differences found in Hue values for *P. abyssynicum* and the rest of species and subspecies with coloured seed is due to different chemical composition or in the oxidative reactions involved in its formation.

Gigantism is another trait associated to the domestication syndrome. It can be assessed by seed calibre (C) and W100S and by pod characteristics as PD and PL. In the present study, *Pisum sativum* subsp. *sativum* had the highest W110S and C and high PL and PD with no significant differences to *P. abyssynicum*, *P. elatius* and *P. sativum* subsp. *jomardii*. The smallest seeds and pod where those from *P. fulvum* and *P. sativum* subsp. *transcaucasicum*, because these specie and subspecie showed the lowest W100S, C, PL and PD.

P. fulvum was the specie with the lowest PH and showed also low DF with no significant differences with *P. abyssynicum*, *P. elatius* and *P. sativum* subsp. *sativum*. These two traits are generally associated to the domestication syndrome but also can be subject to major post-domestication changes during the history of the crop. Flowering in pea is regulated by six gene mediating response to vernalisation and photoperiod (King and Murfet, 1985). The worldwide expansion of the crop may have imposed adaptation to different conditions such as vernalisation response in some regions wile in more temperate (or hot) climes this characteristic had been lost. Also, cultivation practices have made that precocious variants of the crop are preferred in the necessity to fit the crop in a rotation scheme in some regions wile in zones where pea is the only annual crop this characteristic is irrelevant. In the same direction, changes in harvesting techniques (from manual, to reaping and trashing, to completely mechanized) make the plant height a blurred trait to study domestication, as a plant with low height can correspond to the "wild type" (as *P. fulvum*) or to a newly released cultigen adapted to mechanical harvest.

Phylogeny

Makasheva (1979) adopted two species: *P. fulvum* and *P. sativum*, this last one integrated by six subspecies (subsp. *sativum*, subsp. *elatius*, subsp. *transcaucasicum*, subsp. *abyssinicum*, subsp. *asiaticum* and subsp. *syriacum*).

Maxted and Ambrose (2001) and Smýkal (2011) adopted three species: *P. fulvum* Sibth. & Sm.; *P. abyssinicum* A. Br. and *P. sativum* L. integrated with two subspecies: subsp. *sativum* (includes *var. sativum* and *var. arvense*); subsp. *elatius* (Bieb.) Aschers. & Graebn (includes *var. elatius*, *var. brevipedunculatum* and *var. pumilio*).

Martin-Sanz et al. (2011) applied principal component and phylogenetic analyses and established three well defined species in the genus (*P. abyssynicum*, *P. fulvum* and *P. sativum*) while *P. elatius* and *P. humile* couldn't be differentiated.

Vershinin *et al*. (2003) studied the genetic structure and evolutionary history of *Pisum* based on retrotransposon sequence-specific amplification polymorphisms (SSAP) of four main *Pisum* species, *P. fulvum*, *P. elatius*, *P. abyssinicum*, and *P. sativum* and found a distinct pattern of the Neighbour-joining) tree for each basic lineage, which reflects the different evolutionary history of each species. Furthermore, *P. elatius* accessions were separated into two groups carrying contrasting alleles associated with domestication one of them that can be considered the wild forms of *P. elatius*, whereas the other one likely represent the section of *P. elatius* from which the antecedents of cultivated *P. sativum* were drawn and that *P. abyssinicum* and *P. sativum*, arose independently in contrasting ways via the common processes of hybridization, introgression, and selection.

According to the presence of the variants of three functionally unrelated polymorphic markers referring to plastid, mitochondrial and nuclear sequences (rbcL, coxI and SCA, respectively), Kosterin and Bogdanova (2008) found that all accessions of *P. fulvum* and *P. abyssinicum* had combination A, indicating that this is the ancestral state in the genus, while cultivated forms of *P. sativum* had combination B and wild representatives had both combinations A and B, as well as rare combinations that may have resulted from occasional crosses or represent intermediate evolutionary lineages. In this investigation, the authors consider an Egyptian cultivated form '*Pisum jomardii* Schrank', wich is a rare recombinant, as a subspecies, and ascribed formally the name: *Pisum sativum* L. subsp. *jomardii* (Schrank) Kosterin; also all wild forms were considered within a fuzzy paraphyletic subspecies *P. sativum* subsp. *elatius* (Bieb.) Schmalh.

In the present study, the distances among species and subspecies (Table 4·) show that *P. fulvum* had the greatest distance to *P. sativum* subsp. *sativum*, followed by distance to *P. sativum* subsp. *jomardii* while distances to *P. elatius*. *P. abyssynicum* and *P. sativum* subsp. *transcaucasicum* were moderate and similar in magnitude. *P. abyssynicum* showed the greatest distances with all *P. sativum* subspecies, and moderate distance to *P. elatius* and *P. fulvum*, in agreement with the previously cited literature that bring *P. abyssinicum* an specie status in contrast to those that consider it as a *P. sativum* subspecie.

P. elatius showed moderate distances to *P. fulvum*, *P. abyssynicum* and *P. sativum* subsp. *sativum*, while distances to

the "wild" subspecies of *P. sativum (transcaucasicum* and *jomardii*) were the smallest found (3.55 and 3.16 respectively). This fact supports the model proposed by Martin-Sanz et al. (2011), Smýkal et al. (2011 and 2015) and Kosterin and Bogdanova (2008) of a paraphyletic group, *P. sativum* subsp. *elatius*, within which all *P. sativum* are nested.

When a Neighbour-joining tree with 10,000 replicates bootstrap is made (Figure 1), a clade conformed by *P. abyssynicum* accessions with a very strong bootstrap support (88%) can be differentiated from the other two clades, one conformed by *P. fulvum* and one accession of *P. elatius*, and the other one conformed by all subspecies of *P. sativum* and the rest of *P. elatius* accessions. This results are in consensus with Vershinin et al. (2003) who stated that these two independently domesticated pea species arose in contrasting ways from the common processes of hybridization, introgression, and selection.

In the case of *P. elatius*, the Neighbour-joining tree obtained doesn't support a paraphyletic group. Kosterin *et al*. (2010) pointed to the lineage B of *P. sativum* subsp. *elatius* as the origin of the cultivated *P. sativum* while linage A remained as the wild form of the specie. In the present study, only one accession (JI 2629) belonging to linage B according to Kosterin and Bogdanova (2014), appeared in the clade of *P. fulvum*, thus, the traits used here don't have correlation with the distinction of linages inferred by mitochondrial, plastid and nuclear gene studies. Further analysis is needed to elucidate phylogenetic relation among *P. elatius* and *P. sativum*.

Materials and methods

Plant material and experimental design

Ninety-two accessions of *Pisum* from a working collection where studied during 2015 and 2016, in a randomized complete block design with 2 replications, in plots of 1 m^2 (approximately 100 plants) in late June, at the Experimental Field of the College of Agricultural Sciences, Rosario National University, located in Zavalla (33° 1´ S and 60° 53´ W). Irrigation was used until flowering and pod setting stages. The original set of accessions had two representatives of *P. fulvum* specie, two *of P. abyssinicum*, four of *P. elatius* and 83 of *P. sativum*. In order to balance the number of representatives of each specie and to reduce distortion in the analysis due to duplicate or very similar accessions records, a core subset from *P. sativum* was generated applying the advance M strategy with heuristic search using the PowerCore v1.0 software as proposed by Kim et al. (2007). A total of 11 accessions (13.25% of the original set) were selected, with accessions representing every subspecie studied. Comparative values for the means among the original collection and the core set (data not shown) indicate the presence of homogeneity of means between the collections for 10 (71.43%) of the 14 traits analysed. Descriptive parameters of the core collection were: mean difference percentage (MD%) = 10.33%, variance difference percentage (VD%) = 30.99%, variable rate of coefficient of variance (VR%) = 128.1% and the coincidence rate (CR) = 86.39% indicating that the core collection has good representation of its original collection (Hu et al., 2000). The Efficiency Index is indicative of the effectiveness of PowerCore in comparison to the non-heuristic search and was 1. The accessions used in the final analysis, including the core subset of *P. sativum*, is shown in Table 1.

Traits measured

The variables analysed were days until 50% of plants flowering (DF); weight of 100 seeds (W100S) in grams; plant height (PH), calibre of grains (C), pod length (PL) and pod diameter (PD) in centimetres. Colour traits where measured on two-dimensional digital images of 600 dpi taken on a Samsung CLX 3300 scanner of samples of 50 seeds or 20 pods per repetition and analysed using Tomato Analyzer (TA) software (Rodríguez et al., 2010). The colour traits were the two colour coordinates (a and b), as well as a psychometric index of lightness (L) from the CieLab system of colour; the parameters RGB (red, green and blue), the Hue angle value and the Chroma index, where: a coordinate indicates the greenness-redness of the colour (-a is green, +a is red) and varies between -128 and 128; b coordinate indicates blueness-yellowness of the colour (-b is blue, +b is yellow) and varies between -128 and 128; L is an approximate measurement of luminosity, which is the property according to which each colour can be considered as equivalent to a member of the greyscale, between black and white; Chroma is the difference of a hue in comparison to a grey colour with the same lightness (the higher the Chroma values, the higher is the colour intensity of samples perceived by humans) and Hue angle defines the pure colour and mixtures of them in a continuum that range from 0º to 359º. (Hue=0º is red, Hue=60º is yellow, Hue=120º is green, Hue=180º is cyan, Hue=240º is blue and Hue=300º is magenta) and is calculated as: Hue angle = tan-1 (a/b).

Statistical analysis

An analysis of variance (ANOVA) was performed using InfoStat for Windows (Di Renzo et al., 2012). A tree using the Weighted Neighbour-joining Method was made using the DARwing software (Perrier and Jacquemoud-Collet, 2006).

Conclusion

We can conclude that seed and pod characteristics as colour parameters and size, obtained by digital phenotyping, have proved to be suitable markers for genetic diversity evaluation and the construction of core collections with good representation of this variability. They are useful also in evolutionary analysis and variety discrimination, allowing the discrimination of the main wild and cultivated species in the genus *Pisum*.

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References

Abbo S, Pinhasi Van-Oss R, Gopher A, Saranga Y, Ofner I, Peleg Z (2014) Plant domestication versus crop evolution: a conceptual framework for cereals and grain legumes. Trends Plant Sci. 9(6): 351-360.

Bastianelli D, Grosjean F, Peyronnet C, Duparque M, Régnier J M (1998) Feeding value of pea (*Pisum sativum* L) 1 Chemical composition of different categories of pea. Animal Sci. 67: 609-619.

Congling W, Rattandeep G, Ye C, Corley Holbrook C, Ozias-Akins P (2015) Fine phenotyping of pod and seed traits in *arachis* germplasm accessions using digital image analysis. Peanut Sci. 15: 1-26.

Dell' Aquila A (2009) Digital imaging information technology applied to seed germination testing. A review. Agron Sustain Dev. 29: 213–221.

Di Rienzo J A, Casanoves F, Balzarini M G, Gonzalez L, Tablada M, Robledo C W (2012) InfoStat versión 2012 Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL http://wwwinfostatcomar

Dutta Gupta S, Ibaraki Y, Pattanayak AK (2013). Development of a digital image analysis method for real-time estimation of chlorophyll content in micropropagated potato plants. Plant Biotechnol Rep. 7:91–97.

Ellis NTH, Vershinin A, Knox MR, Turner L, Ambrose MJ (2005) Evolutionary change in *Pisum* genome organization. In: Tuberosa R, Phillips RL, Gale M (eds) In the wake of the double helix: from the green revolution to the gene revolution Bologna, Italy, p 91–102.

Fedoruk MJ, Vandenberg A, Bett KE (2013) Quantitative trait loci analysis of seed quality characteristics in lentil using single nucleotide polymorphism markers. Plant Genome. 6(3):10.

Gepts P (2004) Crop domestication as a long-term selection experiment. Plant Breed Rev. 24:1-44.

Hu J, Zhu J, Xu HM (2000) Methods of constructing core collections by stepwise clustering with three sampling strategies based on the genotypic valued of crops. Theor Appl Genet. 101:264-268.

Jing R, Johnson R, Seres A, Kiss G, Ambrose MJ, Knox MR, Ellis THL, Flawell AJ (2007) Gene-based sequence diversity analysis of wild pea (*Pisum*). Genetics. 177:2263-2275.

Kim KW, Chung HK, Cho GT, Ma KH, Chandrabalan D, Gwag JG, Kim TS, Cho EG, Park YJ (2007) PowerCore: A program applying the advanced M strategy with a heuristic search for establishing core sets. Bioinformatics. 23:2155-2162.

King WM, Murfet IC (1985) Flowering in *Pisum*: a sixth locus: Dne. Ann Bot. 56(6):835-846.

Kosterin O, Bogdanova V (2008) Relationship of wild and cultivated forms of *Pisum* L as inferred from an analysis of three markers, of the plastid, mitochondrial and nuclear genomes. Genet Resour Crop Evol. 55:735–755.

Kosterin O, Zaytseva E, Bogdanova VS, Ambrose MJ (2010) New data on three molecular markers from different cellular genomes in Mediterranean accessions reveal new insights into phylogeography of *Pisum sativum* L subsp *elatius* (Bieb) Schmalh. Genet Resour Crop Evol. 57:733–739.

Kosterin OE, Bogdanova VS (2014) Reciprocal compatibility within the genus *Pisum* L as studied in F1 hybrids: 1 Crosses involving *P sativum* L subsp *sativum.* Genet Resour Crop Evol. 62(5):691-709.

Makasheva RK (1979) Gorokh (pea). In: Korovina ON (ed) Cultivated Flora of the USSR. Kolos Publisher Leningrad, p 1-324.

Martin-Sanz A, Caminero C, Jing R, Flavell AJ, Perez De La Vega M (2011) Genetic diversity among Spanish pea (*Pisum sativum* L) landraces, pea cultivars and the World *Pisum sp* core collection assessed by retrotransposon-based insertion polymorphisms (RBIPs). Span J Agric Res. 9(1):166-178.

Maxted N, Ambrose MJ (2001) Peas (*Pisum* L) In: Maxted, N, Bennett, S J (eds) Plant genetic resources of legumes in the Mediterranean, Kluwer Academic Publishers, The Netherlands, p 181–190.

Patwardhan A, Ray S, Roy A (2014) Molecular markers in phylogenetic studies-A Review. J Phylogenetics Evol Biol. 2(2):1-10.

Perrier X, Jacquemoud-Collet JP (2006) DARwin software http://darwinciradfr/darwin

Porob S, Naik G, Velingkar H, Amonkar D, Patil R and Bhat P (2017) Plant Health Monitoring using Digital Image Processing. IJETED. 7(3): 147-151.

Rodríguez GR, Moyseenko JB, Robbins MD, Morejón NH, Francis DM, Van Der Knaap E (2010) Tomato Analyzer: a useful software application to collect accurate and detailed morphological and colorimetric data from two-dimensional objects. J Vis Exp 37. http://dxdoiorg/103791/1856

Smulikowska S, Pastuszewska B, Święch E, Ochtabińska A, Mieczkowska A, Nguyen VC, Buraczewska L (2001) Tannin content affects negatively nutritive value of pea for monogastrics. J Animal Feeds Sci. 10:511-523.

Smýkal P, Coyne CJ, Ambrose MJ, Maxted N, Schaefer H, Blair MW, Berger J, Greene SL, Nelson MN, Besharat N et al (2015) Legume crops phylogeny and genetic diversity for science and breeding: Critical Review Plant Sci. 34(1-3):43-104.

Smýkal P, Jovanovic Z, Stanisavljevic N, Zlatkovic B, Cupina B, Dordevic V, Mikié A, Medovic AA (2014) Comparative study of ancient DNA isolated from charred pea (*Pisum sativum* L) seeds from an Early Iron Age settlement in southeast Serbia: inference for pea domestication. Legumes Perspectives. 5:9–10.

Smýkal P, Kenicer G, Flavell AJ, Corander J, Kosterin O, Redden RJ, Ford R, Coyne CJ, Maxted N, Ambrose MJ, Ellis THL (2011) Phylogeny, phylogeography and genetic diversity of the *Pisum* genus. Plant Genet Res. 9:4-18.

Stanek M, Zduńczyk Z, Purwin C, Florek S (2004) Chemical composition and nutritive value of seeds of selected pea varieties. Vet Zootech Lith. 28(50):71-74.

Troszynska A, Ciska E (2002) Phenolic compounds of seed coats of white and coloured varieties of pea (*Pisum sativum* L) and their total antioxidant activity. Czech J Food Sci. 20:15-22.

Vershinin AV, Allnutt TR, Knox MR, Ambrose MJ, Ellis TH (2003) Transposable elements reveal the impact of introgression rather than transposition, in *Pisum* diversity, evolution and domestication. Mol Biol Evol. 20:2067-2075.

Vollmann J, Walter H, Sato T, Schweiger P (2011) Digital image analysis and chlorophyll metering for phenotyping the effects of nodulation in soybean. Comput Electron Agric. 75: 190-195.

Weeden N (2007) Genetic Changes Accompanying the Domestication of *Pisum sativum*: Is there a common genetic basis to the 'Domestication Syndrome' for legumes? Ann Bot. 100:1017-1025.

Xiaofang W, Warkentin TD, Briggs CJ, Oomah BD, Campbell CJ, Woods S (1998) Total phenolics and condensed tannins in field pea (*Pisum sativum* L) and grass pea (*Lathyrus sativus* L). Euphytica. 101:97-102.

Zohary D, Hopf M (2000) Domestication of plants in the Old World 3rd edn New York: Oxford University Press, p 1-316.