Bulletin of the National Research Centre

RESEARCH Open Access

Karyotype variation and biochemical analysis of five *Vicia* species

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Abstract

Background: Fabaceae is considered as the third largest family, which includes more than 727 genera and 20,000 species. The genus *Vicia* has from 180 to 210 species. *Vicia* species have a great economical and agricultural importance. Karyotype study of chromosomes and SDS-PAGE for seed storage proteins (soluble and non-soluble proteins) were carried out on five *Vicia* species (*Vicia macrocarpa*, *Vicia sativa*, *Vicia narbonensis*, *Vicia ervilia*) collected from IPK, Germany, and *Vicia faba* from Agriculture Research Centre, Giza, Egypt, to find out the phylogenetic relationships among these species.

Result: From karyotype of studied *Vicia* species chromosomes, it was found that *V. macrocarpa*, *V. sativa*, and *V. faba* had six pairs of chromosome ($2n = 12$) while *V. narbonensis* and *V. ervilia* had seven pairs of chromosome ($2n = 14$). The most related species was found between *V. ervilia* and *V. narbonensis* (77.8%) depending on seed soluble protein similarity level, but between *V. narbonensis* and *V. macrocarpa* was 70.0% depending on seed non-soluble protein similarity level, while between *V. ervilia* and *V. narbonensis*, the most related species was 69.0% depending on collective data of both soluble and non-soluble seed storage protein.

Conclusion: The phylogenetic relationships between the studied species depending on collective data of protein markers and karyotype characteristic were as follows: *V. ervilia* is closely related to *V. narbonensis*, while *V. narbonensis* is related to *V. macrocarpa* and *V. ervilia*, but the degree of relation between *V. narbonensis* and *V. macrocarpa* is less than the relation between *V. narbonensis* and *V. ervilia*. Equally, while *V. sativa* is closely related to *V. macrocarpa*, but *V. faba* is distant from all other studied species.

Keywords: *Vicia* species, Karyotype, SDS-PAGE, UPGMA

Introduction

Fabaceae is considered the second family after cereal crops in agricultural importance based on area harvested and total production; this family contains more than 727 genera and 20,000 species (Gepts et al. 2005). The species in genus *Vicia* (180 to 210 species) are widely distributed throughout the world. This genus has two subgenera, *Vicia* and *Vicilla*, and the subgenus *Vicilla* is considered more primitive and diverse than the subgenus *Vicia* (Hanelt and Mettin 1989; Maxted 1993). The subgenus *Vicilla* is divided into 17 sections including forage species. Kupicha (1976) suggested that the subgenus *Vicia* is smaller and coherent, containing 38 species divided into 5 sections. This subgenus contains the more agriculturally important species of *V. faba* (section Faba), *V. sativa* (section Vicia), and *V. narbonensis* (section Narbonensis).

Karyological studies had an important role in improvement and solving taxonomic problems between the related species (Lavia et al. 2009; Murti et al. 2012). The cytogenetic comparisons based on chromosome size, centromeric index, and banding patterns between related species occurred by staining chromosomes with different dyes such as feulgen, orcein, or carmine

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(Cremonini 1992; Galasso et al. 1994; Cremonini et al. 1998; Fuchs et al. 1998).

Until recently, cytotaxonomic relationships between species were performed using conventional staining methods to visualize the chromosomes. The development and application of banding techniques for plants have proved to be a practical tool for identifying chromosomes as well as providing much information regarding species relationships. The most popular staining procedures include Q, G, C, R, and silver stain banding which have been developed for bright-field microscopy (Casperson et al. 1970; Howell et al. 1975; Fominaya et al. 1988; Jellen et al. 1993; Jellen and Ladizinsky 2000).

Polyacrylamide gel electrophoresis (PAGE) plays an important role in the analysis of protein profile. PAGE is considered the most widespread technique. Seed storage proteins were used in investigating genetic diversity and evaluation of taxonomic and genetic associations in *Vicia* (Emre et al. 2010).

The aim of our research is to find out the phylogenetic relationships between five *Vicia* species (*V. macrocarpa* (Moris) Betol., *V. sativa* (L.) ssp. *Sativa* convar var. *sativa*, *V. narbonensis* (L.) var. *narbonensis*, *V. ervilia* (L.) Willd, and *V. faba* var. sakha 3) by studing the karyotype of chromosomes and seed storage protein (soluble and non-soluble proteins) profile of the studied species.

Materials and methods

All the laboratory experiments were carried out in the laboratories of Genetics and Cytology Department, Genetic Engineering and Biotechnology Research Division, National Research Centre, Giza, Egypt.

Plant materials

Seeds of four *Vicia* species such as *V. macrocarpa* ((Moris) Betol.), *V. sativa* L. (ssp. *Sativa convar* var. sativa), *V. narbonensis* L. (var. *narbonensis*), and *V. ervilia* L. (Willd) were obtained from the germplasm collection of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, except for *V. faba* (var. sakha 3) from the Agricultural Research Center, Giza, Egypt.

Chromosome preparation

The seeds of *Vicia* species were germinated on moist filter paper at 20 °C. Root tips of about 1–2 cm length were excised. The roots were treated with ice-cold water for 20–22 h to arrest the chromosome at metaphase then fixed in Carnoy's solution I (3:1 v/v) absolute ethanol to glacial acetic acid for 24 h at 4 °C, then stored in a refrigerator in 70% ethanol; after that, the roots were incubated in 1% cellulose and 1% pectinase (v/v) which were dissolved in 0.01 M citrate buffer pH 4.8 at 37 °C for 1 h. Root tips were squashed on slides in a drop of 45% acetic acid, frozen on liquid nitrogen to remove the coverslips. After that, slides were washed with Carnoy's fixative solution and air dried. The slides were stained with DAPI; after that, they were examined under florescence microscope.

Karyotype characteristics

After photos were captured with a camera connected to a computer, the chromosomes of each cell were arranged using the Adobe Photoshop 6.0 software. After finishing the arrangement of chromosomes of one species, a computer program (Micromeasure 3.3) was used to measure the total length of each chromosome, length of the short arm, length of the long arm, arm ratio (long/short), centromeric index [short/(long + short)], and the relative length (RL) for each chromosome (percentage of total length of haploid complement). The ideograms for the 5 species were drawn in Corel-Draw program.

The procedure of Bebeli and Kaltsikes (1985) was followed to describe the chromosome types in the five species according to the location of the centromere, i.e., metacentric to cover the M-chromosomes with an arm ratio (S:L) between 1:1.35, submetacentric to cover the Sm chromosomes with an arm ratio between 1.36 and 1.75, and subtelocentric to cover the St-chromosomes with an arm ratio greater than 1.76.

Seed storage protein profiles using SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method proposed by Laemmli (1970), as modified by Studier (1973). Water-soluble proteins (WSP) and water-non-soluble proteins (WNSP) were extracted from the seed of five *Vicia* species.

Results

Karyotype characteristics

The somatic chromosome number of two species (*V. narbonensis* and *V. ervilia*) is $2n = 14$, while in the other three species (*V. sativa*, *V. macrocarpa*, and *V. faba*) is $2n = 12$. All of the examined species have one secondary constriction except for *V. sativa* which has two. For karyotypic analysis, chromosomes were captured by a cooled CCD camera and analyzed on a computer with image analysis software (Photoshop 0.6). Chromosomes were randomly numbered, and the total length and lengths of the short arm (S) and long arm (L) were measured for each chromosome. Using chromosome-measuring software, the short and long arms of the homologous chromosome pairs were measured and identification based on chromosome arm ratio (L/S). In

the karyogram construction, chromosome pairs were ordered from longest to shortest based on the relative length of each pair of chromosomes. At least 10 well-spread chromosome preparations of each species were analyzed to validate the karyogram construction for each species. The total length and the length of the short arm, long arm, arm ratio, the relative length of each chromosome, and the chromosome types of the five *Vicia* species are depicted in Table 1. The karyotype of the five *Vicia* species and their ideograms are shown in Fig. 1.

Vicia macrocarpa

The length of the short arms for the different chromosomes ranged from 7.1 ± 0.83 to 2.47 ± 0.61 μm, whereas the long arms gave average lengths from 9.54 ± 1.21 to 4.44 ± 0.35 μm. Chromosomes no. 1, 5, and 6 are metacentric, having arm ratios of 1.23 ± 0.18, 1.09 ± 0.08, and 1.29 ± 0.21, respectively, while chromosomes no. 2 and 3 are submetacentric, having arm of ratio 1.71 ± 0.39 and 1.65 ± 0.21, respectively. Chromosome no. 4 is subtelocentric, having arm ratio of 3.68 ± 1.08. Chromosome no. 6 has the secondary constriction region on its relatively short arm.

Vicia sativa

The length of the short arms for the different chromosomes ranged from 4.72 ± 0.78 to 1.66 ± 0.21 μm, whereas the long arms gave average lengths from 7.78 ± 1.01 to 3.39 ± 0.29 μm. The chromosome nos. 2, 3, and 5 are metacentric, having arm ratios of 1.18 ± 0.85, 1.29 ± 0.46, and 1.35 ± 0.25, respectively. Chromosome nos. 1, 4, and 6 are subtelocentric, having arm ratios of 2.15 ± 0.38, 2.06 ± 0.60, and 2.07 ± 0.26, respectively.

Vicia narbonensis

The length of the short arms for the different chromosomes ranged from 5.99 ± 0.5 to 4.72 ± 0.57 μm, whereas the long arms gave average lengths from 10.36 ± 0.46 to 6.24 ± 0.52 μm. The chromosome no. 7 is metacentric, having arm ratio of 1.22 ± 0.14%, while chromosome no. 4 is subtelocentric, having arm ratio of 1.76 ± 0.19. Chromosome nos. 1, 2, 3, 5, and 6 are submetacentric, having arm ratios of 1.74 ± 0.16, 1.72 ± 0.26, 1.65 ± 0.26, 1.72 ± 0.26, and 1.55 ± 0.26, respectively.

Vicia ervila

The length of the short arm for the different chromosomes ranged from 7.73 ± 0.46 to 1.98 ± 0.19 μm, whereas the long arms gave average lengths from 8.88 ± 0.33 to 6.13 ± 0.19 μm. Chromosome nos. 1, 2, and 6 are metacentric, having arm ratios of 1.14 ± 0.1, 1.26 ± 0.16, and 1.31 ± 0.1, respectively. Chromosome no. 3 is submetacentric, having an arm ratio of 1.46 ± 0.16. Chromosome nos. 4, 5, and 7 are subtelocentric, having arm ratios of 2.23 ± 0.07, 2.44 ± 0.36, and 3.12 ± 0.40, respectively. Chromosome no. 7 has the secondary constriction region on its long arm.

Vicia faba

The length of the short arms for the different chromosomes ranged from 11.82 ± 1.01 to 1.52 ± 0.25 μm, whereas the long arms gave average lengths from 13.24 ± 1.43 to 8.69 ± 0.74 μm. Chromosome no. 1 is metacentric and has an arm ratio of 1.12 ± 0.12. Chromosome nos. 2, 3, 4, 5, and 6 are subtelocentric, having arm ratios of 7.38 ± 1.18, 6.38 ± 2.04, 2.48 ± 0.42, 6.66 ± 0.72, and 5.4 ± 1.2, respectively.

Seed storage protein profiles using SDS-PAGE

Seed storage proteins (soluble and non-soluble proteins) fractions were used to find out the relationships between the studied five *Vicia* species (Fig. 2).

The studied *Vicia* species were examined for seed storage protein profile, and the data was subjected to unweighted pair-group method with arithmetical average (UPGMA) to find the phylogenetic relationships among the species. Table 2 and Fig. 3 represented the relationship between the studied *Vicia* species depending on seed soluble protein similarity level, and it was found as follows: *V. ervilia* and *V. narbonensis* are the most related

Table 1 The principal characteristics of the chromosomes of the haploid complement of five *Vicia* species

Chromosome number	*Vicia macrocarpa*				*Vicia sativa*				*Vicia narbonensis*				*Vicia ervilia*				*Vicia faba*			
	S	L	AR	CT	S	L	AR	CT	S	L	AR	CT	S	L	AR	CT	S	L	AR	CT
I	7.10	8.61	1.23	M	3.70	7.78	2.15	St	5.99	10.36	1.74	Sm	7.73	8.76	1.14	M	11.82	13.24	1.12	M
II	5.73	9.54	1.71	Sm	4.72	5.61	1.18	M	5.57	9.44	1.72	Sm	7.07	8.88	1.26	M	1.71	12.46	7.38	St
III	4.39	7.19	1.65	Sm	4.48	5.80	1.29	M	5.41	8.78	1.65	Sm	5.69	8.29	1.46	Sm	1.82	10.94	6.38	St
IV	2.47	8.55	3.68	St	2.97	5.78	2.06	St	4.89	8.58	1.76	St	3.79	8.47	2.23	St	3.58	8.69	2.48	St
V	5.10	5.57	1.09	M	3.70	4.88	1.35	M	4.74	8.04	1.72	Sm	3.47	8.43	2.44	St	1.52	10.02	6.66	St
VI	3.50	4.44	1.29	M	1.66	3.39	2.07	St	4.72	7.24	1.55	Sm	5.10	6.66	1.31	M	1.69	8.97	5.4	St
VII									5.17	6.24	1.22	M	1.98	6.13	3.12	St				

S short arm, *L* long arm, *AR* arm ratio, *CT* chromosome type, *St* subtelocentric chromosome, *M* metacentric chromosome, *Sm* submetacentric chromosome

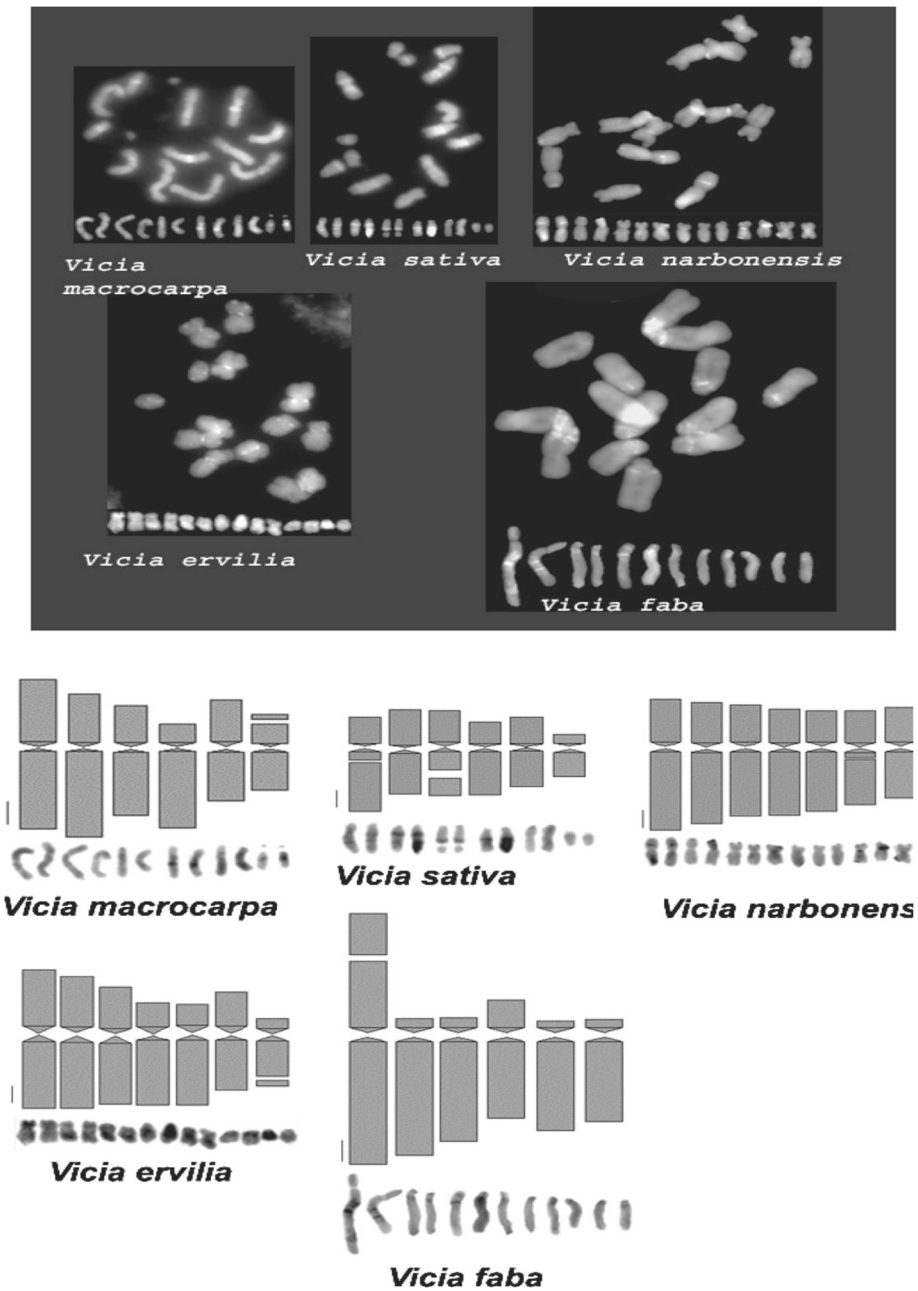

Fig. 1 Karyotype and idiograms of *V. macrocarpa*, *V. sativa*, *V. narbonensis*, *V. ervilia*, and *V. faba* (scale = 2 μm)

species (77.8%), then comes *V. macrocarpa* which is most related to *V. narbonensis* (64.5%), *V. sativa* which is most related to *V. macrocarpa* (60.0%), and peripheral position comes *V. faba* which has almost the same relationship with the other studied species, while Table 3 and Fig. 4 showed the relationship between the studied five species depending on seed non-soluble protein similarity level, and it was found as follows: *V. narbonensis* and *V. macrocarpa* are the most related species (70.0%), then comes *V. sativa*, *V. ervilia*, and *V. faba*.

The collective data of both soluble and non-soluble seed proteins were analyzed using UPGMA (Table 4 and Fig. 5); the relationship between the studied five species was as follows: *V. ervilia* and *V. narbonensis* were the most related species (69.0%), then *V. narbonensis* and *V. macrocarpa* (67.2%), *V. macrocarpa* and *V. sativa* (60.7%), and *V. faba* at a peripheral position.

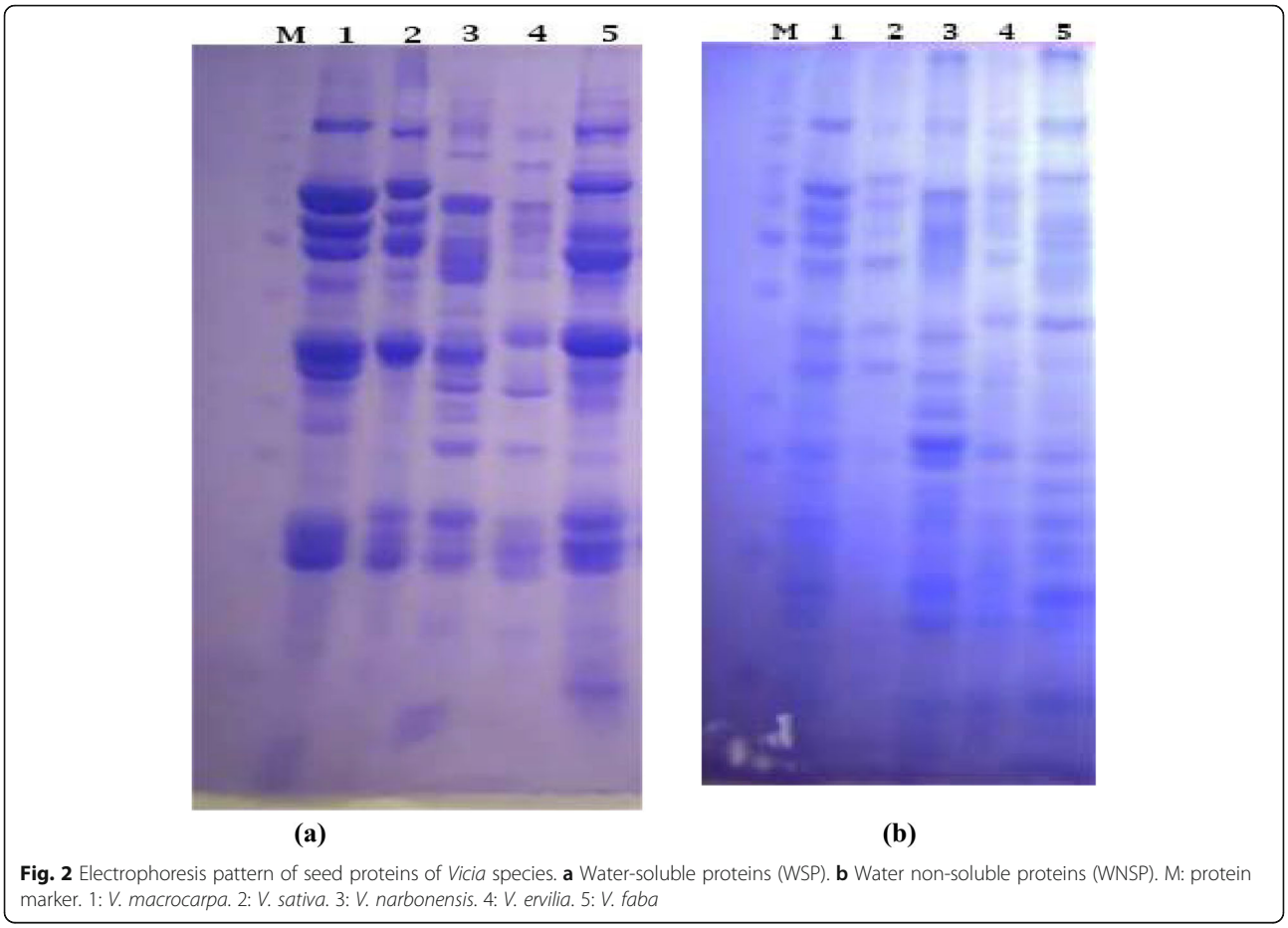

Fig. 2 Electrophoresis pattern of seed proteins of *Vicia* species. **a** Water-soluble proteins (WSP). **b** Water non-soluble proteins (WNSP). M: protein marker. 1: *V. macrocarpa*. 2: *V. sativa*. 3: *V. narbonensis*. 4: *V. ervilia*. 5: *V. faba*

Discussion

The karyotypes of several species have been established based on chromosome size and centromeric index in addition to the traditional process for karyotyping by adding a dye to metaphase chromosomes. Different dyes that affect different areas of the chromosomes are used for a range of identification purposes. One common dye used is Giemsa; this dye is effective because it markedly stains the bands on a chromosome; each chromosome can then be identified by its banding patterns (Cremonini 1992; Galasso et al. 1994; Cremonini et al. 1998; Fuchs et al. 1998); however, this approach is limited by the similar morphology of chromosomes in many species.

Chromosome features and their count have been recorded in cytological characterization of germplasm (Sharma and Sharma 2013). The genus *Vicia* become an interesting model for studying a plant genome and karyotype evolution due to the variation in basic chromosome number between *Vicia* species $2n$ = 10, 12, or 14 (Maxted 1995). El-Bok et al. (2014) mentioned that the chromosome numbers varied between *Vicia* species and subspecies such as *Vicia cordata* had $2n$ = 10, *Vicia angustifolia* had $2n$ = 12, *Vicia narbonensis*, and *Vicia monantha* ssp. *calcarata* and ssp. *cinerea* presented $2n$ = 14. Both *V. sativa* ssp. *amphicarpa* accessions with aerial and underground pods showed $2n$ = 14 and were first reported. Chromosome numbers of *V. sativa* ssp. *sativa*

Table 2 The level of similarities among *Vicia* species, produced by Jaccard's coefficient, based on water-soluble proteins

Vicia macrocarpa	1.000				
Vicia sativa	**0.600**	1.000			
Vicia narbonensis	**0.645**	0.545	1.000		
Vicia ervilia	0.552	**0.552**	**0.778**	1.000	
Vicia faba	0.548	0.548	0.545	**0.552**	1.000
	Vicia macrocarpa	*Vicia sativa*	*Vicia narbonensis*	*Vicia ervilia*	*Vicia faba*

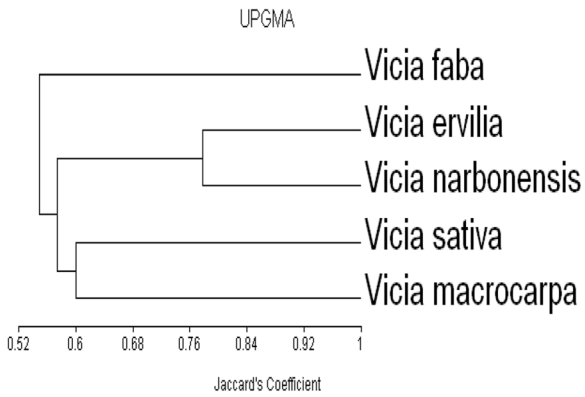

Fig. 3 UPGMA dendrogram of *Vicia* species based on frequencies of seed soluble proteins

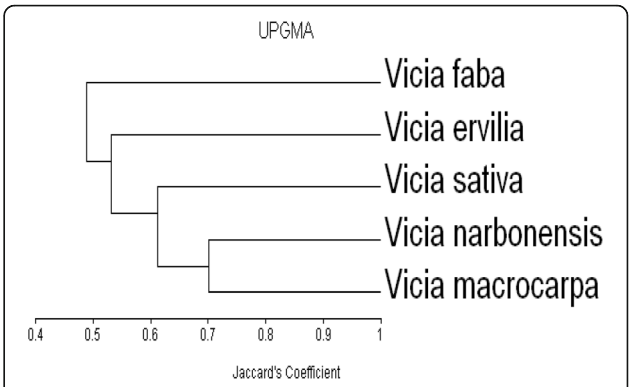

Fig. 4 UPGMA dendrogram of *Vicia* species based on frequencies of seed non-soluble proteins

were verified and revised as $2n$ = 10, 12. Also, Gaffarzadeh-Namazi et al. (2008) found that *Vicia* species from Iran were different in chromosome number, karyotype formula, and karyotype characteristics such as *Vicia villosa* ($2n = 2x = 14$), *Vicia hyrcanica* ($2n = 2x = 12$), *V. sativa* subsp. *sativa* ($2n = 2x = 12$), and *V. sativa* subsp. *nigra* ($2n = 2x = 12$).

In our results, *V. macrocarpa* ((Moris) Betol.) had six pairs of chromosomes ($2n = 12$): three metacentric, two submetacentric, and one subtelocentric chromosome; this nearly agrees with Raina et al. (2001) who worked on *Vicia macrocarpa* ((Moris) Arcang).

V. sativa L. (ssp. *Sativa convar*. var. sativa) had six pairs of chromosomes ($2n = 12$): three metacentric and three subtelocentric chromosomes; this results are agreement with the results of Davis and Plitmann (1970), Raina and Rees (1983), and Maxted et al. (1991) who found that the chromosome numbers for *V. sativa* (subsp. *sativa*) and *V. sativa* (subsp. *macrocarpa*) were $2n = 12$, while there are different chromosome number reported in *V. sativa* (subsp. *Incise* var. incise) ($2n = 14$) in the study of Çiler and Feruzan (1999). But it was determined to be $2n = 10$ for *V. sativa* subsp. *incisa* var. *cordata* as reported by Raina and Rees (1983) and Kamari et al. (1994). *V. sativa* subsp. *nigra* was found to have $2n = 12, 14$ (Davis and Plitmann 1970; Tutin 1968), while *V. sativa* subsp. *Amphicarpa* had $2n = 14$ (Tutin 1968).

V. narbonensis L. (var. *narbonensis*) had seven pairs of chromosomes ($2n = 14$): one metacentric, one subtelocentric, and five submetacentric chromosomes; this is in agreement with the result of Navrátilová et al. (2003) who worked on *Vicia narbonensis* (L.) IFYN574, and with the results of Raina et al. (2001) who worked on *V. narbonensis* (ssp *narbonensis*).

In our results, *V. ervilia* L. (Willd) had seven pairs of chromosomes ($2n = 14$): three metacentric, one submetacentric, and three subtelocentric chromosomes, while *V. faba* (var. sakha 3) had six pairs of chromosomes ($2n = 12$): one metacentric and five subtelocentric chromosomes which disagree with Hizume et al. (1980) in chromosome type; they studied the C-banding patterns on *V. faba* using Giemsa stain and found that the number of chromosomes $2n = 12$ (one metacentric, three subtelocentric, and two telocentric); the metacentric chromosome associated with secondary constriction on short arm.

SDS-PAGE considered a genetic markers in analyses of genetic distances between species to determine the taxonomic relationship (Tamkoc and Arslan 2011).

In our study, the five *Vicia* species were examined for protein profile levels and the data was subjected to unweighted pair-group method with arithmetical average (UPGMA) to find the phylogenetic relationships among the species. First, the relationship between the studied five *Vicia* species depending on seed soluble protein

Table 3 The level of similarities among *Vicia* species, produced by Jaccard's coefficient, based on water non-soluble proteins

Vicia macrocarpa	1.000				
Vicia sativa	**0.615**	1.000			
Vicia narbonensis	**0.700**	**0.607**	1.000		
Vicia ervilia	0.567	0.414	**0.613**	1.000	
Vicia faba	0.441	0.483	0.529	**0.500**	1.000
	Vicia macrocarpa	*Vicia sativa*	*Vicia narbonensis*	*Vicia ervilia*	*Vicia faba*

Table 4 The level of similarities among *Vicia* species, produced by Jaccard's coefficient, based on collective date of seed water-soluble proteins and water non-soluble proteins

Vicia macrocarpa	1.000				
Vicia sativa	**0.607**	1.000			
Vicia narbonensis	**0.672**	**0.574**	1.000		
Vicia ervilia	0.559	0.483	**0.690**	1.000	
Vicia faba	0.492	0.517	0.537	**0.525**	1.000
	Vicia macrocarpa	***Vicia sativa***	***Vicia narbonensis***	***Vicia ervilia***	***Vicia faba***

similarity level was as the follows: *V. ervilia* and *V. narbonensis* are the most related species (77.8%), then comes *V. macrocarpa* which is most related to *V. narbonensis* (64.5%), and *V. sativa* which is most related to *V. macrocarpa* (60.0%), and peripheral position comes *V. faba* which has almost the same relationship with the other studied species (Table 2 and Fig. 3). Second, the relationship between the studied five species depending on seed non-soluble protein similarity level was as the follows: *V. narbonensis* and *V. macrocarpa* are the most related species (70.0%), then comes *V. sativa*, *V. ervilia*, and *V. faba* (Table 3 and Fig. 4), while the relationship between the studied five species depending on the collective data of both soluble and non-soluble proteins using UPGMA was as the follows: *V. ervilia* and *V. narbonensis* were the most related species (69.0%), then comes *V. narbonensis* and *V. macrocarpa* (67.2%), then comes *V. macrocarpa* and *V. sativa* (60.7%), and *V. faba* at a peripheral position (Table 4 and Fig. 5).

There are a few studies made to determine the genetic diversity between faba bean and its related *Vicia* species (Haider et al. 2001). In that study, 13 taxa representing 6 *Vicia* species (*V. sativa*, *V. villosa*, *V. monantha*, *V. narbonensis*, and *V. cinerea*, in addition to *V. faba*) were collected from the north-west coastal region of Egypt. SDS-PAGE indicated clear differences between different *Vicia* species as well as within the taxa of the same species. The results indicated that *V. monantha* and *V. villosa* are most closely related to *V. cinerea*, *V. faba*, and *V. narbonensis*, which are completely distant. The marked differences between *V. faba* and other wild species indicated that none of the latter can be considered as the wild progenitor of *V. faba*, while Kahraman et al. (2016) implicated that protein banding patterns for investigating 22 bean genotypes provided a clear classification by view of selection criteria. Similarity dendrogram presented two main groups that showed ranges nearly 20–75% and 50–90%, respectively.

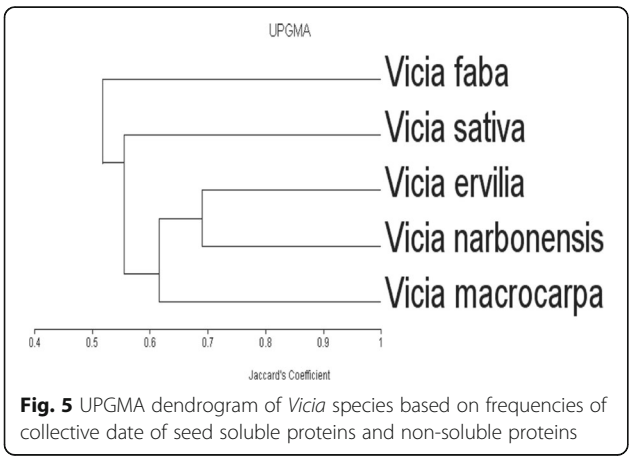

Fig. 5 UPGMA dendrogram of *Vicia* species based on frequencies of collective date of seed soluble proteins and non-soluble proteins

Conclusion

The phylogenetic relationships among the studied species depending on the seed storage proteins profile (soluble and non-soluble proteins) using SDS-PAGE and karyotype characteristic were as follows: *V. ervilia* was closely related to *V. narbonensis*, and *V. narbonensis* was related to *V. macrocarpa*, but the degree of relation between *V. narbonensis* and *V. macrocarpa* was less than the relation between *V. narbonensis* and *V. ervilia* which were the most related species, while *V. sativa* was closely related to *V. macrocarpa*, but *V. faba* was distant from all other studied species. This study helps the breeder to perform the breeding program without consumption of a long duration for doing several crossing over for doing a hybridization between different species.

Abbreviations

S: Short arm; L: Long arm; S:L: Arm ratio; Sm: Submetacentric; St: Subtelocentric; M: Metacentric; RL: Relative length; UPGMA: Unweighted pair-group method with arithmetical average; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WSP: Water-soluble protein; WNSP: Water non-soluble protein

Acknowledgements

I would like to thank the Genetics and Cytology Department, National Research Centre, because this research work was done in the Genetic and Cytology Laboratory.

Authors' contributions

Authors SAO and HBA designed the study and managed the laboratory experiments and analyzed all data. Authors ZME and SEE managed the literature searches and wrote this manuscript. All authors approved the final manuscript.

Funding

This research was not funded by any funding body.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 29 August 2019 Accepted: 1 June 2020
Published online: 09 June 2020

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