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REGULAR ARTICLE

Phylogeny of Orchidaceae species in northwest Syria based on ISSRs.

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ABSTRACT

Syria is a rich country with many orchid species of the family *Orchidaceae* which has a considerable economic importance in horticulture, floristry and the pharmaceuticals and fragrance industries. There is, however, a disagreement among taxonomists regarding the classification of the family at the genus and species levels. Moreover, there are few orchid species described recently in Syria to which no flora of the country has pointed out. Hence, there is a great need to revise, using molecular markers, the classifications available for orchid species distributed in Syria which are based solely on morphological characterisation. The aim of this study, therefore, was to carry out a morphological and molecular characterisation of orchid species distributed in the North Western region of Syria which is very rich with these species. A set of 34 plant samples that represent all orchid species observed in the region of study were analysed using inter-simple sequence repeats (ISSRs) to resolve genetic relationships among species targeted. The dendrogram constructed using the unweighted pair group method with arithmetic averages (UPGMA) and percent disagreement values of the Statistica program showed that species were grouped in two main clusters. The first cluster included all samples of the genus *Ophrys*, while all remaining species were grouped in the second cluster in which species that belong to the controversial genus *Orchis* were overlapped with other genera. Based on results generated, the classification status of some *Orchis* species was revised. Revealing phylogenetic relationships among species studied will guide hybridization and genetic engineering programs applied on those species.

Keywords: characterisation, molecular, orchids, phylogenetic, relationships

INTRODUCTION

In Syria and Lebanon, there are about 3650 vascular plant species that belong to more than 130 family and 910 genera [1]. Syria is regarded as the origin of many wild plant species that are distributed naturally in the country such as olives, figs and almond, beside many fruity forest trees and crops such as wheat, barley and chickpeas. The Syrian flora is also very rich with medical (e.g. *Mellissa officinalis*, *Origanum syriacum* and *Scilla maritima*) and ornamental (e.g. *Lonicera etrusca*, *Iris ssp.* and *Jasminum fruticans*) species [2].

Wild relatives of plant species which are of a high economic value provide valuable genetic resources that can be utilised for the improvement of these species. There are many wild plant species, however, that are neglected and not exploited for the development of new cultivars. Examples of those wild species are those of the family *Orchidaceae*, commonly referred to as the

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orchid family that is still neglected in Syria in spite of its wide distribution. Furthermore, some Syrian orchid species are endangered of extinction.

The family Orchidaceae

The family *Orchidaceae* belongs to the monocotyledons; division *Magnoliophyta*, class *Liliopsida* and order *Asparagales* [3]. The family includes five subfamilies on which there is a broad agreement: *Apostasioideae*, *Cypripoideae*, *Epidendroideae*, *Orchidoideae* and *Vanilliodeae* [4, 5]. Freudenstein and Rasmussen [6] reported that *Orchidaceae* is the largest family of angiosperms (ca. 10% of all flowering plants and ca. 1/3 of the monocots, 7]. It contains perennial grasses, some of which live on the soil, some others on trees in tropical regions as Epiphytic, and some others on the degradation of organic materials as Saprophytic. The family is believed to be clearly monophyletic and of a relatively ancient origin (probably Early Cretaceous) as suggested by molecular clock estimates [8, 9].

The *Orchidaceae* is one of the most species-rich plant families [10]. It includes about 800 genera and 25000 species [11, 7], most of which are in the tropical regions, and is renowned for its many diverse, even bizarre, specialized pollination systems [12]. Due to the diversity of specialized pollination and ecological strategies of *Orchidaceae* species, Freudenstein and Rasmussen [6] believe that the family provides a rich system to study evolutionary patterns. According to Ramírez et al. [13], *Orchidaceae* is the most diverse plant family on Earth but many aspects of their evolutionary history still obscure because it lacks a definitive fossil record. Results generated by the authors supported an ancient origin for *Orchidaceae* and revealed that the most recent common ancestor of extant orchids lived in the Late Cretaceous (76-84 Myr ago).

Orchid species have a very high economic value because of their flowers that are used widely for ornamental purposes (the industry being worth more than $110 million annually in the US alone, 7]. Added to that, the Vanillia is extracted from the genus *Vanilla* and Sahlab from the tubers of the genus *Orchis* [14]. There are also many species of the family that have medical uses such as *Vanilla planifolia* (stomach activation and preparation of some medicines), and those of the genus *Orchis* that have various uses for diaries and as activators and pain relievers in enteritis for children [15]. Recently, transformation and regeneration of plants via genetic engineering have a powerful tool for orchid improvement [16].

Distribution of the Orchidaceae family

The *Orchidaceae* occurs in almost every habitat apart from glaciers and deserts, and most orchid species are found in the tropics (mostly Asia, South America, and Central America) and above the Arctic Circle, in southern Patagonia and even on Macquarie Island, close to Antarctica [3].

Orchid species are adapted to all environmental conditions ranging form rainy forests to dry deserts, and from sea level to the top of mountains where there are no trees [17]. In Syria, Mouterde [1] and Post [18] mentioned that orchid species are distributed in the Western region from the North (Aleppo and Idleb) to the South (Goulan and Horan etc.) and the middle area (Hama and Homs). Orchid species are also observed in Damascus and the surrounding regions such as Guta, Qasion, Deemas and Bloudan, as well as in the Costal region stating from sea level to the highest mountain tops. Due to the large number of those species, the lack of a fossil record, and a historical emphasis on characters related to floral morphology, Chase [19] believes that until recently orchid classification has been poorly understood.

Classification of orchid species of Syria

Post [18] mentioned that the *Orchidaceae* family is represented in Syria with 45 species that belong to nine genera. Whereas, Mouterde [1] believes that there are eight genera that are represented by 31 species (Table 1a, b). Comparison of the two classifications presented in Table 1, shows that there is a disagreement in the classification view of the orchid species in Syria at the species and genus levels by the two taxonomists. Aswad [20] also mentioned three other new orchid species that exist in Syria and to which none of the floras available for Syrian referred. These are: *Cephalathfra damasonium* (Mill) Druce., *Spiranthes autumnalis* (Baip) L.C.Rich, and *Neotinea intacta* (Link) Reichbg.

Table 1. Syrian genera and species of *Orchidaceae* according to flora of Post [18] (a) and Mouterde [1] (b).

a)

Genus	No. of species	Genus	No. of species
Gymnadenia	1	*Serapia*	2
Cephalanthera	4	*Loroglpssum*	2
Epipactis	2	*Anacamptis*	1
Limodorum	1	*Orchis*	18
		Ophrys	14

b)

Genus	No. of species	Genus	No. of species
Ophrys	8	Limodorum	1
Orchis	14	*Cephalanthera*	3
Anacamptis	1	*Epipactis*	2
Serapias	1	*Platanthera*	1

From what stated above, a classification revision of the orchid species grow in Syria at the molecular level should be made via revealing genetic relationships among the species considered. The utility of the PCR-based Inter-Simple Sequence Repeat (ISSR) variations as phylogenetic markers for investigating evolutionary relationships among plant species has been clearly established [21, 22]. Thus, in the present study, the genetic relationships among species studied were determined using the ISSRs technique. However, it is a hard task to cover all orchid species of Syria in one study. Therefore, only those species that are distributed in the North-West region of Syria (Allepo, Idlib, and Lattakia) were considered because this region is the richest region with orchid species in Syria.

MATERIALS AND METHODS

Plant material and DNA extraction

Plant material was collected from 30 orchid species from all regions of northwest Syria that were referred to in available Syrian floras to have orchids (Table 2). Leaf samples were collected from these species that were identified based on flower morphology as described in the flora of Mouterde [1] since it was the last flora that screened the region. Collected leaves were washed three times in sterile distilled water, immersed in liquid nitrogen, and kept at -60 ^{o}C until use. For the molecular analysis, more than one sample was used for species *Oph. fuciflora* and *Oph. attica* (three samples for each), and *Oph. bornmuelleri* (two samples) (Table 2) because there is 1) variation within each of those species either in flower color (*Oph. bornmuelleri* and *Oph. fuciflora*) or plant size (*Oph. attica*) and 2) disagreement of the different floras on the classification of these species since some floras pointed out that different flower color or plant size represents different species. DNA was isolated by a modified quick method of Dorokhov and Klocke [23]. A weight of 0.5 g of young leaves was ground to powder in liquid nitrogen using a mortar and a pestle. The powder was transferred into 2 ml Eppendorf tubes and mixed with 800 µl of extraction buffer [200 mM Tris-HCl (pH=7.5), 250 mM NaCl, 25 mM Na_2EDTA (ethylenediamine

Table 2. Names of orchid species studied, and Syrian regions from which those species were collected (classification according to The Kew World Checklist of Monocotyledons, as slightly modified by Florida Museum of Natural History [7]).

Sample no.	Tribes or subtribes	Orchid species	Region collected from
1	*Orchidinae*	*Ophrys fuciflora* (Crantz) Haller	Samaan castle, Allepo
2	*Neottieae*	*Limodorum abortivum* (L) Sw.	Haj Husnali, Allepo
3	*Orchidinae*	*Ophrys bornmuelleri* M.Schulze	Al-Shoyokh, Allepo
4	*Orchidinae*	*Orchis italica* Poiret	qatmah, Allepo
5	*Orchidinae*	*Ophrys fuciflora*	Samaan castle, Allepo
6	*Orchidinae*	*Serapias vomeracea* (Burm.) Breiquet	Harim, Idlib
7	*Orchidinae*	*Ophrys bornmuelleri*	Om Al-Tyoor, Latakia
8	*Orchidinae*	*Ophrys attica* (Boiss. et Orph.) Soo`	Khan Al-aasal, Allepo
9	*Orchidinae*	*Orchis collina* Banks et Sol.	Khan Al-aasal, Allepo
10	*Orchidinae*	*Orchis tridentata* Scop. var.commutata (Tod.) Rchb.f.	Om Al-Tyoor, Latakia
11	*Orchidinae*	*Ophrys sp*	Iki Dam , Allepo
12	*Orchidinae*	*Orchis sancta* L.	Harim, Idlib
13	*Orchidinae*	*Orchis iberica* Bieb.	Azar, Idlib
14	*Orchidinae*	*Ophrys scolopax* Cav.	Azar, Idlib
15	*Orchidinae*	*Himantoglossum affine* (Boiss.) Schlecht.	Bab Al-Hawa, Idlib
16	*Orchidinae*	*Platanthera chlorantha* (Custer) Reichb.	Al-Furunloq, Latakia
17	*Spiranthinae*	*Spiranthes autumnalis* (Balb.) L.C. Rich.	Om AlTyoor, Latakia
18	*Neottieae*	*Epipactis latifolia* (L.) All.	Azar, Idlib
19	*Neottieae*	*Cephalanthera kurdica* Bornm.	Haj Husnali, Allepo
20	*Orchidinae*	*Ophrys attica* (the bigger size)	Mroneh, Allepo
21	*Orchidinae*	*Orchis morio* L. ssp. picta (Loisel.) Reichb., var. libani Renz	Kasab, Latakia
22	*Orchidinae*	*Ophrys fuciflora* (White flower)	Samaan castle, Allepo
23	*Orchidinae*	*Ophrys ferrum-equinum*	Samaan castle, Allepo
24	*Orchidinae*	*Ophrys sintenesii* Fleischm. et Bornm.	Iki Dam , Allepo
25	*Orchidinae*	*Ophrys lutea*(Gouan) Cav. ssp. galilaea (Fleischm. et Bornm.) Soo`	Arab Boran, Allepo
26	*Orchidinae*	*Orchis laxiflora* Lam. , ssp. dielsiana Soo`	Ain Dara, Allepo
27	*Neottieae*	*Cephalanthera longifolia* (Huds.) Fritsch	Haj Husnali, Allepo
28	*Orchidinae*	*Ophrys fusca* Link	Harim, Idlib
29	*Orchidinae*	*Orchis anatolica* Boiss.	Al-Shoyokh, Allepo
30	*Orchidinae*	*Ophrys attica* var. orientalis (Renz) n. comp.	Arab Boran, Allepo
31	*Orchidinae*	*Ophrys argolica* Fleischm.	Al-Shoyokh, Allepo
32	*Orchidinae*	*Orchis romana* Seb. et Mauri	Al-Furunloq, Latakia
33	*Orchidinae*	*Anacamptis pyramidalis* (L.) L. C. Rich.	Azar, Idlib
34	*Neottieae*	*Epipactis consimilis* Don.	Hazyrain valley, Latakia

tetra acetic acid), and 0.5% SDS (sodium dodecyle sulfate)]. The tubes were homogenized for 40 seconds, vortexed for 5 seconds, and then incubated at 65 ^{o}C for 15 min. A volume of 400 µl of cold 5M

potassium acetate was added to each tube and put on ice for 10 min. The tubes were then centrifuged at 12000 rpm for 15 min, and the upper aqueous phase (about 500 µl) was recovered to a new tube and mixed with the same volume of isopropanol and left for 10 min. The tubes were centrifuged at 12000 rpm for 10 min, and the nucleic acid precipitate was washed twice with cold 65% ethanol, the tubes were then centrifuged at 12000 rpm for 10 min. Recovered DNA pellets were dried under the laminar flow and then resuspended in 150 µl of doubled distilled and sterilized water. DNA was quantified using Gene Quant Spectrometer (Amersham Biosiences, UK) and the concentration of all samples was set at 10 ng/µl.

ISSRs analysis and interpretation of data generated

Using 19 selected primers ([24, 25], Table 3), ISSR analysis [26] was carried out on orchid samples used. The amplification was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 m $Mg(OAc)_2$, 0.2 mM of each dNTP (Roche), 1 mM $MgCl_2$, 0.5U of Taq DNA polymerase (Eurobio), 15 ng of genomic DNA, 125 Pmol from each primer (Invitrogen), 10.5 µl 2 % formamide (Eurobio), and 1.2 µl of 10 mM spermidine (Fluka). Samples were subjected to an initial denaturation at 94 ^{o}C for 2 min, followed by 40 cycles, each consisting of 94 ^{o}C for 10 sec, 45-60 ^{o}C (depending on the GC content of the primer, Table 3) for 10 sec, and 72 ^{o}C for 10 sec. A final extension at 72 ^{o}C was carried out for 10 min. Amplification products were stored at 4 ^{o}C before analysis.

Amplification products generated were size separated by standard horizontal electrophoresis in a 2.5% agarose (Merck) gel (to which ethidium bromide (Fluka) was added) in a 0.5X (TBE) buffer. Electrophoresis was performed at 85 V for 2.30 hours. A 1 Kb DNA ladder (Fermentas) was used to estimate the approximate molecular weight of DNA bands for each amplification product.

Amplification profiles generated from ISSRs were screened and photographed under UV light. All reactions were repeated at least twice and only bright and reproducible bands were scored as 1 (present) and 0 (absent). The unweighted pair group method with arithmetic averages (UPGMA) and percent disagreement values of the Statistica program were used to construct the matrices and the dendrogram [27].

RESULTS

Based on the flora of Mouterde [1], it was a difficult task to identify orchid species that grow in the region screened. Due to the high homology in the vegetative part among orchid species, it was also not possible to identify the different species that belong to the same genus when there were no flowers on the plant. However, it was easier to identify the plants at the genus level.

Using 19 ISSR primers, 34 samples of orchids were analysed for molecular variation. A total number of 439 band lines was obtained, all of which (100%) were polymorphic. The total number of bands in all lines was 2207. Table 4 shows the total number of bands generated from the use of each primer and the number of polymorphic bands obtained from the respective primers.

All ISSR primers showed polymorphism among species that ranged from19 (primers A-4 and C-22) to 32 (C-26) polymorphic lines with an average of total lines number of 23 for all primers. The size of amplification

Table 3. Primers used for ISSR analysis, their nucleotide sequence and annealing temperatures.

Primer name	Nucleotide sequence (5`-3`)	Annealing temperature (^{o}C)
A1	CACACACACACARR	50
A4	CACACACACACARY	50
A16	CACACACACACAR	50
A37	AGCAGCAGCAGCM	60
A40	AGCAGCAGCAGCM	50
A44	AGCAGCAGCAGCS	55
B1	CTC TCT CTC TCT CTC TTG	50
B4	CAC ACA CAC ACA GG	50
C24	CTCTCTCTCTCTCTCTTG	50
A31	AGCAGCAGCAGCR	50
B16	GAC AGA CAG ACA GAC A	50
B7	GTG GTG GTG GC	50
A34	AGCAGCAGCAGCY	50
C27	GATA GATA GATA GATA	50
C22	AGAGAGAGAGAGAGAGT	58
C26	CACACACACACAGG	58
C25	GTGGTGGTGGC	60
B10	CAG CAG CAG CAG CAG	50
A7	CACACACACACARM	50

Table 4. Number of polymorphic lines and bands generated using ISSRs on samples analysed.

Primer name	No. of polymorphic lines	No. of bands generated
A1	29	148
A4	19	71
A16	25	100
A37	19	88
A40	20	79
A44	22	87
B1	24	121
B4	28	189
C24	21	73
A31	20	125
B16	22	146
B7	23	82
A34	20	84
C27	25	84
C22	19	76
C26	32	213
C25	25	163
B10	20	109
A7	26	169

fragments ranged from 90 to 1100 bp. The PDV matrix (data not shown) constructed had PDV values that ranged from 0.06 (between samples *Oph. sintenesii* and *Oph. ferrum-equinum,* and *Oph. attica* (the smaller size) and *Oph. bornmuelleri* (sample no. 7)) to 0.29 (between samples *Oph. fusca* and *O. italica,* and *Oph. bornmuelleri* (sample no. 7) and *C. kurdica*). These data are in total conformity with the dendrogram constructed (Figure 1).

As for the dendrogram constructed for samples analysed using ISSRs data generated, two main clusters were observed. The first cluster included all samples of the genus *Ophrys* and the remaining species were grouped in the other cluster. *Ophrys* species were separated into three clades. The first clade had *5Oph. fuciflora* and three combinations of sister species: *1Oph. fuciflora* and *3Oph. bornmuelleri*, *7Oph. Bornmuelleri* and *8Oph. attica*, and *20Oph. attica* and *22Oph. fuciflora.* Species *Oph. ferrum-equinum, Oph. sintenesii*, *Oph. attica* var. orientalis and *Oph. argolica* were grouped in the second clade. The remaining two species *Oph. lutea* and *Oph. fusca*, were shown as sister species in the third clade of the *Ophrys* cluster. Species in the second cluster were grouped in two clades; the first had species *O. laxiflora*, *O. romana* and *A. pyramidalis*, and the second included the remaining samples apart from species *S. autumnalis*, *O. anatolica*, *O. tridentata* and *O. italica* that were the most distant from other species in the cluster. Within this group, *L. abortivum* was the most distant. Species clustered in this group were divided into two clades. The first clade included only three species: *O. laxiflora*, *A. pyramidalis* (as sister species) and *O. romana*. In the second clade, the two species of each of the genera *Cephalanthera* and *Epipactis* were sister species. The *Orchis* species *O. sancta* and *O. iberica* had the same scenario. The dendrogram also showed that *S. vomeracea* was grouped with *P. chlorantha*, *O. morio* was the closest to *H. affine*, and *O. collina* was the most distant from the remaining species in this group.

DISCUSSION

Chase et al. [5] argued that the circumscription of genera, subtribes, tribes, and subfamilies and the relationships among them in previous classifications of the family *Orchidaceae* are unclear due to the homoplasy in morphological and anatomical features. The authors stated that the most widely used orchid classification, at present, is that of Dressler [28]. Rothacker [29] believes that this classification is one of the most comprehensive classifications to date, although it (as all other morphological characters-based classifications) was often based on a few so-called key characters [5] and relied heavily on vegetative and floral morphology that ecological selection may make them express considerable convergence [30]. Chase et al. [5] also argued that this classification and the more recent one of Szlachtko in 1995 are clearly out-of-date relative to the cladistic analyses of Neyland and Urbatsch [31], Chase et al. [32], Cameron et al. [4] and Freudenstein and Rasmussen [6]. Therefore, a new subfamilial classification of *Orchidaceae* was proposed by the authors.

As for revealing phylogenetic relationships among orchid species, orchids have now been the focus of more published DNA phylogenetic studies than any other family in the angiosperms [5]. Since *Orchidaceae* was first classified by Swartz (1800; 1805) (and more completely by Lindley (1830-40) and others), the phylogeny of the family has undergone a number of revisions (cited in [29]).

The first attempt, however, to reconstruct phylogenetic relationships across the whole family of *Orchidaceae* based on morphological description-based cladistic analysis was that of Burns-Balogh and Funk

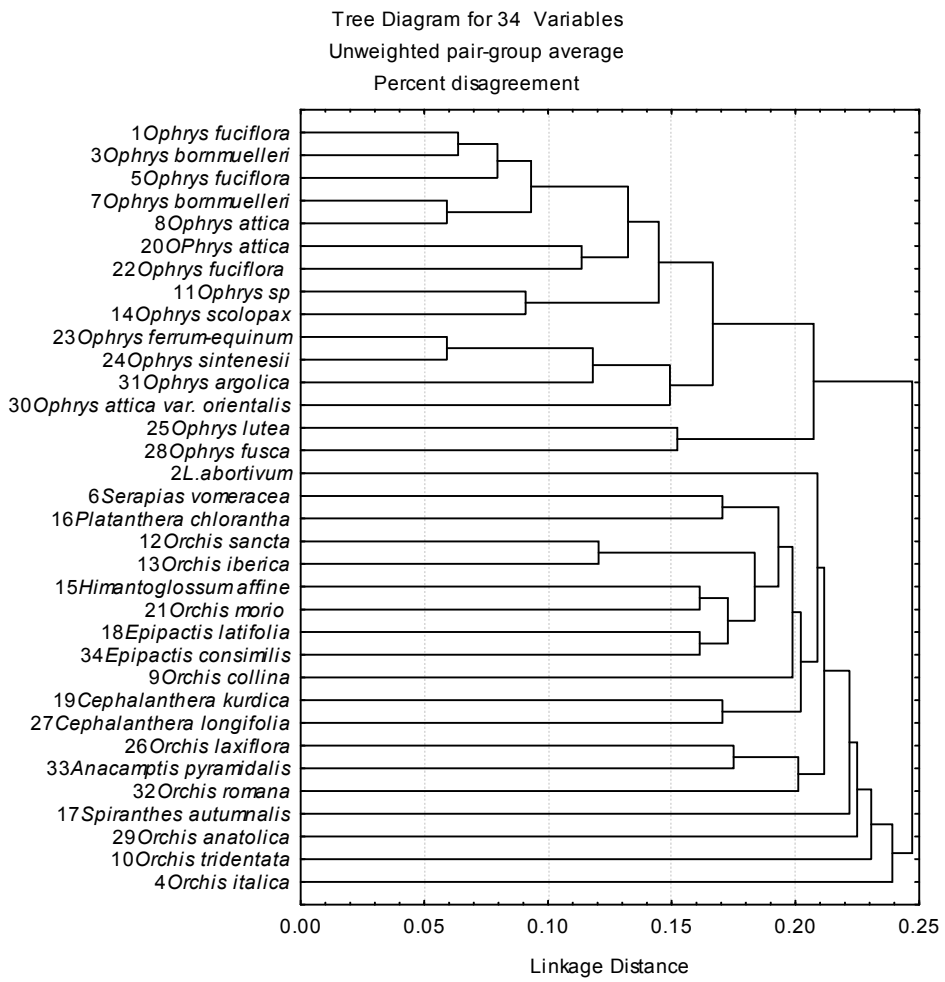

Figure 1. The dendrogram constructed for the 34 orchid samples based on the ISSRs-based PDVs of UPGMA, Statistica.

[33]. This was followed by the study of Freudenstein and Rasmussen [6] that was also based on morphological characters analyzed by cladistic methods. Florida Museum of Natural History [7] believes that although the resolutions of the trees generated by both studies were poor, these studies demonstrated the value of such information, especially with regard to translating the orchid phylogeny into a working classificationn. To date, there are, however, several DNA-based phylogenies published for *Orchidaceae* as those carried out on the whole family [31, 34, 32, 4, 35- 39] or subfamilies [40- 42].

At the tribe and subtribe levels, molecular phylogeny of the tribe *Diseae* (*Orchidoideae*, *Orchidaceae*) based on nrITS1, 5.8S rDNA, and ITS2 sequences was constructed [43, 30]. In 2001, Kores et al. [44] carried out a phylogenetic analysis on the tribe *Diurideae* based on plastid DNA sequence data. In a similar study, the monophyly of and phylogenetic relationships within the orchid tribe *Maxillarieae* Pfitzer were evaluated based on combined nuclear ribosomal and plastid DNA sequence data of ITS1 and ITS2, *matK*, the *trnL* intron and the *trnL-F* intergene spacer [45]. Bateman et al. [46] also studied the subtribe *Orchidinae* based on nrITS. Recently, Bytebier et al. [47] inferred phylogenetic relationships for the African subtribe *Disinae* based on sequences of nrITS

gene region and two plastid regions (*trnL-F* and *matK*). Based on restriction site analysis of the chloroplast DNA, the phylogeny of the subtribe *Dendrobiinae* was determined by Yukawa et al. [48]. For more examples on other orchid tribes and subtribes phylogenetics, see Chase et al. [5].

Some other studies on orchids focused on revealing phylogenetic relationships among or within genera such as *Coelogyne* [49, 50], and *Orchis* and allied genera (*Anacampti, Himantoglossum*, *Neotinea, Ophry,* and *Platanthera)* [51]. The classification of the genus *Himantoglossum* was also reviewed by Delforge [52]. Very recently, Bellusci et al. [53] determined phylogenetic relationships in the genus *Serapias* based on noncoding regions of the chloroplast genome. The molecular phylogeny of the genus *Aerides* (*Orchidaceae*) based on one nuclear and two plastid markers was constructed by Kocyan et al. [54]. Parker and Koopowitz [55] determined the classification status of species of *Disa oripetaloides* group. The phylogenetic species concept for the species *Corallorhiza macalata* was also studied by Freudenstein and Doyle [56]. Similarly, species-level evolutionary and classification questions have been investigated using the nITS region (combined often with data from the plastid genome) by, for instance, Bateman et al. [46] and Clements et al. [57].

It is worth noting that most systematic work on the family has been concentrated at the species level, describing the seemingly boundless variation that occurs particularly in tropical regions [6]. Combining such morphological analysis, however, with molecular analysis is expected to yield a more informative system for revealing relationships among species. It should be noted that this is the first study that evaluates the phylogenetic relationships of the wild species of the orchid family in the region of study (North-West Syria) at the molecular level. In the present study, we analyzed the nDNA using ISSRs in 34 orchid samples collected from the North-West region of Syria that represented all species found in the region, and for which we have developed species-specific CAPS markers [58]. The aim of this study was to relieve the phylogenetic relationships among the species considered. The study also aimed at comparing the molecular characterization of the species studied with their morphological characterization.

Phylogenetic tree constructed here showed that all *Ophrys* species were clustered in one clade [59]. This agrees with the classifications of available flora of those species, all of which reveal high homology in morphological characters and with the fact that they share unique characters such as the absence of spur of the flower, the shape of the Labellum that resembles, generally, the bee shape, the smaller petals than sepals, and the shorter bracts than the flowers. This is also supported by the findings of Davis and Huxley [60] that all members of the genus *Ophrys* have a distinct morphology that makes it unique and clearly different from the remaining species of the family. We can conclude from the clustering all *Ophrys* species in one clade that this genus is monophyletic.

A very close genetic relationship was revealed among the five samples of *Oph. bornmuelleri* and *Oph. fuciflora* that represented the different colours and regions of the two species since they were grouped in one clade. This is congruent with all morphological characterization-based floras, some of which consider the different phynotypes of each of those species as subspecies of the same species.

As for the species *Oph. attica*, the phylogenetic tree revealed that it is the closest species to the previous two species. This agrees with the morphological description of the flora of Mouterde [1] that demonstrates the high homology among these species. The high morphological similarity between *Ophrys sp* and *Oph. scolopax* as the

morphological characterization showed was supported by the high genetic homology between the two species since they were revealed to be sister species in the tree constructed here. Regarding the variety *Oph. attica* var. orientalis, it has been shown to be remarkably genetically different from the other two samples that belong to same species *Oph. attica.* This agrees with the view of Royal Botanic Gardens in Kew/London (a personal communication) that this species is a separate species called *Ophrys umbilicata.*

A close genetic relationship was observed between *Oph. argolica* and *Oph. sintenesii*. They are also very similar morphologically since the Labellum is convex with no excrescence, and the big flowers with no side segments in both species. However, they differ in that the Labellum in *Oph. argolica* has colored spots like horseshoeshaped, whereas the Labellum is more curvy and the spots appear as two parallel lines in *Oph. sintenesii.*

The tree also revealed that the species *Oph. ferrum-equinum* is very genetically close to the species *Oph. sintenesii* and *Oph. argolica*. This is supported by the high morphological homology between these species. However, *Oph. ferrum-equinum* differs from the other two species in that the Labellum is black and it has a shiny horseshoe-shaped spot.

The two species *Oph. fusca* and *Oph. lutea* were revealed to be sister species and the most distant from remaining species in the *Ophrys* cluster. This is supported by the morphological description of the two species since the Labellum has no excrescence and has an edge with a lighter colour than the middle part and this is a unique character in these two species.

Devey et al. [61] stated that highly variable, yet possibly convergent, morphology and lack of sequence variation have severely hindered production of a robust phylogenetic framework for the genus *Ophrys.* The authors believe that some putative *Ophrys* species arose through hybridization rather than divergent speciation.

As for the remaining species, they were grouped in one clade that is separated from that of the genus *Ophrys.* The species *L. abortivum* is distant from the other genera in the same clade. This is supported by the morphological description of the genus *Limodorum* that is morphologically different from all other genera studied in that it does not have leaves, and it grows Saprophytic.

The phylogenetic tree also revealed that the four species belonging to the genera *Epipactis* and *Cephalanthera* are very similar at the molecular level and this is supported with the high morphological homology between the two genera since they both differ from the remaining orchid genera in that their root is rhizome while it is tuberous in the remaining genera.

As for the species *Sp. autumnalis*, it is genetically distant from the remaining genera. This species is also distinct for few morphological characters such as the spike which arises from a point close to the point of emergence of the leaves, while in the other genera it appears from the middle and it may carry some leaves. This genus also does not flower every year as the remaining genera do.

Regarding the species *S. vomeracea,* it was shown to be genetically distant from the remaining species. This is supported with the morphological uniqueness of this species in that the flower has both of the Hypochil and Epichil. The closest species of the remaining species to *S. vomeracea* is *P. chlorantha.* This is supported by the high homology between the two species in their flower morphology.

The phylogenetic tree revealed that the species *H. affine* is the closest to the genus *Orchis*. This agrees with the morphological characters-based classification of these species since the roots are tuberous and the Labellum is long and segmented in *H. affine* as in some *Orchis* species.

Davis and Huxley [60] pointed out that species of the genus *Orchis* show many differences in the morphology among the different species belong to it, but they share some characters. They also consider this genus as unique in morphology and hard to classify [62] in the family. As for species of the genus *Orchis* studied here, we noticed in the ISSRs-based tree an overlap between some of species from the genus with other genera of the family as previous studies pointed out [62].

Regarding the two species *O. iberica* and *O. sancta*, they are highly closely related. This confirms that they belong to the same genus [1] although Kew Gardens regarded them as species from two separate genera (*Dactylorhiz*a *iberica* and *Anacamptis sancta*, respectively).

The species *O. romana* was shown to be distant from other species in the genus. This species was reclassified by the Kew Gardens (personal communication) and regarded it as *Dactylorhiza romana*. It was not possible in this study to revise the classification status of this species accurately due to the absence of any species of the genus *Dactylorhiza* from the plant material studied.

The results also showed that the species *O. tridentata* is the most genetically distant in the genus group. This species was reclassified by the Kew Gardens (personal communication) and regarded as *Neotinea tridentate.* It was not, however, possible to revise the classification status of this species accurately due to the absence of any species of the genus *Neotinea* from the plant material studied.

The tree revealed that *O. laxiflora* is the closest to *Anacamptis pyramidali*s. This agrees with Kew Gardens view that this species is *Anacamptis laxiflora.* The species *O. collina* showed to be distant from most *Orchis* species. This species was reclassified by Kew Gardens experts on orchids and was included within the genus *Anacamptis.* This also applies on *O. morio.* In order to determine the classification status of these two species, more species of the genus *Anacamptis* should be included in the study.

As for the two species *O. anatolica* and *O. italica,* they are the most distant from all other species in the genus. They are the only two species that were grouped in *Orchis* by Kew Gardens experts.

Determination of phylogenetic relationships among orchid species analysed here using molecular data can be regarded as a useful tool that helps evaluate previous classifications that were based solely on morphology for species analysed. It is also valuable as a guide for hybridization in breeding of those species and for genetic engineering programs.

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