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DNA BARCODING SUPPORTS AN EASIER IDENTIFICATION OF ALIEN PLANTS: THE CASE OF THE GENUS *PHYSALIS* (SOLANACEAE) IN THE IBERIAN PENINSULA (SPAIN)

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Abstract - In the present study, two species of the genus *Physalis*, *P. acutifolia* and *P. angulata*, are reported for the first time as casual aliens for Spain and, even for Europe, in the case of *P. acutifolia*. Their identification was based on *a priori* DNA barcoding study to reveal their taxonomic identification, which was followed by *a posteriori* morphological analysis to support the barcoding identification. As many authors have reported, the reduced and even inexistent information from local or regional Floras makes it difficult to distinguish those unreported taxa from distant geographical areas. Therefore, the successfully obtained results would highlight the importance of using an integrative taxonomic approach for further unidentified alien plant species. Detailed descriptions of both species and a dichotomous key of the alien taxa of *Physalis* are provided to facilitate their morphological identification.

Keywords: alien species; European flora; floristic records; genetic markers; morphology; Spain; Solanaceae.

Introduction

Most regions of the world have been colonized by species from many other parts of the globe. The accurate identification of the allochthonous flora is nowadays a fundamental challenge to facilitate the knowledge of the alien species around a given geographical area. Alien plants, and specifically invasive plants, have aroused attention globally for causing negative impacts on the native biodiversity, among other effects, and the design of adequate management plans for avoiding their uncontrolled dispersal might be crucial. However, it can be extremely challenging to rapidly and accurately identify these species only with morphological characters because they are an assemblage of many different families and most plant material lacks enough diagnostic characteristics (Xu et al., 2018). Besides, the allochthonous flora is mainly composed of a specific number of families (e.g. Asteraceae, Amaranthaceae, Cactaceae, Fabaceae, Solanaceae, Poaceae) whose taxonomy is rather complicated. Pyšek et al. (2013) already stated that taxonomic tasks related to determine the correct name of an organism are crucial for implementing effective quarantine measures, monitoring invasions and their invasion pathways, and ensuring that the time to first detection for new invaders is minimized. However, taxonomic identification of alien plants is a hard process that demands expert taxonomists and time, and it is often difficult to distinguish species only with morphology, especially for those unreported taxa originated in distant geographic areas. Pyšek (2003) already remarked the importance of available and updated information about alien flora, which is usually quite reduced, and even nonexistent from local or regional published Floras (note the capital letter to denote published work). Moreover, other aspects related to the specific time of collection (flowering or fruiting period), the scarcity of plant material and their conservation as preserved material often originate a lack of diagnostic morphological features, which also increases the complexity for their satisfactory taxonomic identification.

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DNA barcoding is an effective tool for identifying species using standard DNA regions or barcodes (Hebert et al., 2003; CBOL Plant Working Group, 2009). DNA allows a rapid species discovery and identification and has been widely used for taxonomic identification by targeting known gene regions that permit to discriminate among species. The DNA of an unidentified sample is queried against a reference sequence database and the identity of the matching sequence is assigned to the sample. Despite the described limitations of this molecular tool (Chase & Fay, 2009), it has proved to be functional in the detection of species with high phenotypic plasticity and in early or incomplete developed specimens (Valentini et al., 2008). Among other scopes, DNA barcoding has been recently successfully applied in plant invasion biology (Ghahramanzadeh et al., 2013) and in the identification of invasive species of a specific family or a geographical area (Zhang et al., 2013; Xu et al., 2018). A correct taxonomic identification will lay a solid foundation for further actions (Xu et al., 2018), and therefore, the application of DNA barcoding would be crucial to help and support the taxonomists about the final identification of those new alien species, especially if the available plant material is also somehow incomplete or the preserved material does not show clear diagnostic characters (Ghorbani et al., 2017; Xu et al., 2018).

Based on field surveys along the river Vinalopó (District Medio Vinalopó 38º 28' 41''N 0º 47' 24''W, southeast Iberian Peninsula, Spain), two alien specimens belonging to the genus *Physalis* L. (Solanaceae) have been recently found. According to the last phylogenetic approach of the Solanaceae tribe Physalideae (Deanna et al., 2019), this genus includes about 96 species with a Neotropical distribution, with centres of diversity in Mexico, United States and Central America. *Physalis* is typified by its solitary yellow or white flowers with dark maculations and its fruit completely enclosed by an inflated and accrescent calyx (Pretz & Deanna, 2020). Other closely related taxa, characterized by their fruiting calyx, have also been considered within *Physalis* according to previous taxonomic treatments, as it happens to the monotypic genus *Alkekengi* Mill. This genus is well characterized by bright orange to red papery calyx, and its unique species, *A. officinarum* Moench, appears as *P. alkekengi* L. in many Floras. Many *Physalis* species and close related taxa have largely been cultivated for ornamental or food uses in warm areas worldwide, and therefore, ten alien taxa have been mentioned for European and North African territories (Valdés, 2012). In the Iberian Peninsula (Spain), Sanz-Elorza & Sobrino (2012) mentioned the presence of four taxa of the genus *Physalis* (*P. ixocarpa* Brot., *P. peruviana* L. and *P. philadelphica* Lam., including *A. officinarum* as *Physalis*), which are scattered around this territory. The species boundaries within *Physalis* are sometimes poorly defined (Whitson & Manos, 2005; Deanna et al., 2019) and some taxa are typically defined by certain morphological characters, which are adequately observable on fresh plant material (e.g. the number of angles in fruit, color of spots in the throat). However, and depending on the period of the plant collection, some preserved material could not include certain characters that are only well developed during the flowering or fruiting period (e.g. colour of the veins of the fruiting calyx for *P. philadelphica*). In fact, Sullivan (2004) already stated that some specimens of *Physalis* are difficult to determine from preserved material.

Regarding the collected plant material, European and Spanish Floras were examined to detect previous indications of these species. None of the available literature about *Physalis* taxa around Spain and some close Mediterranean countries concur with the observed morphological features of the collected and preserved material. Actually, certain morphological aspects detailed by the literature are not fully coincident, which led to certain reservations about their taxonomic identification. Due to this situation, integrative taxonomy would be the adequate approach to identify the unknown plant species. Integrative taxonomy is a multidisciplinary discipline that combines evidence from independent data sources (e.g. morphology, DNA, ecology, etc.) in order to reveal biological features relevant for species delimitation (Dayrat, 2005). The combination of different characteristics helps to identify taxa from different environmental data at generic and species levels (e.g. De Mattia et al., 2012; Darienko et al., 2015), using DNA barcoding analyses together with conventional morphological identifications. Molecular techniques serve as a complement to classical morphological analyses to give a final taxonomic identification especially for non-native plant species under any stage of their life cycle (e.g. seedlings), since standard morphological techniques alone are sometimes highly laborious and require taxonomic expertise (Whitehurst et al., 2020). To our knowledge, this study will comprise the first approach to identify alien flora based on integrative taxonomy, and this protocol might be a useful route for further unidentified alien plant species, especially if they belong to complex taxa.

Materials and Methods

DNA amplification and molecular analyses

In order to identify the two unknown *Physalis* samples by DNA barcoding, DNA amplifications (PCR) of the nuclear internal transcribed spacer (ITS) were conducted, since this region has recently been used in this genus for DNA barcoding and phylogenetic studies with notable phylogenetic resolution (Feng et al., 2016; Deanna et al., 2019). Plant material of the two unknown *Physalis* samples was sequenced from the preserved specimens at the ABH

herbarium (University of Alicante, Spain; see Appendix 1) since no new field material was available for this study.

The PCR amplifications were directly conducted with the Thermo Scientific Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific Inc, Waltham, MA, USA), following the Dilution and Storage protocol as described by the manufacturer, using dried plant material (leaves and flowers) from the two different specimens (named as Novelda and Elda, respectively; see Appendixes 1 and 2). The ITS primers used were ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Cycling protocol was an initial denaturation of 98 ºC for 5 min; 35 cycles of 98 ºC for 5 s, 60 ºC for 5 s and 72 ºC for 20 s; and a final extension of 72 ºC for 1 min. PCR products were cleaned using UltraClean PCR Clean-up Kit (MoBio Laboratories Inc, Carlsbad, CA, USA), and sequenced bidirectionally by Macrogen Spain (Madrid, Spain). The obtained sequences were submitted to NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (see Appendix 2).

Firstly, the taxonomic identification of the two unknown *Physalis* sequences was based on the comparison with the reference data deposited in the molecular NCBI GenBank database, using the BLAST algorithm (Altschul et al., 1990). The output results yielded a list of the species name showing the top nearest matches (minimum e-value) according to Meiklejohn et al. (2019) guidelines. Identifications were considered reliable if several records with the same taxonomic name showed similar top match statistics. Conversely, if multiple records with different taxonomic names had the same maximum nearest scores, the identification was treated as an ambiguous match.

Secondly, and following the taxonomic treatment for the genus *Physalis* based on Deanna et al. (2019), a search for the available ITS sequences of *Physalis* species was performed on NCBI GenBank on 25 February 2020 (Appendix 2). Sequences of the species *Alkekengi officinarum* and *Calliphysalis carpenteri* (Riddell) Whitson were used as outgroup according to the phylogenetic treatment of Solanaceae tribe Physalidae (see Zamora-Tavares et al., 2016; Deanna et al., 2019). The sequences were aligned with ClustalW with default settings and posterior manual editing in MEGA X v. 10.1.7 (Kumar et al., 2018). Those sequences too short, too divergent or with too many ambiguities were finally deleted. The ITS aligned matrix consisted of 128 sequences with a length of 690 bases, that came from 13 different origins according to GenBank records (eight from published articles and five from unpublished works; see Appendix 2 for details). Neighbour-Joining (NJ) trees were constructed with the Maximum Composite Likelihood model, uniform rates among sites, pairwise deletion in the gaps, and, similarly to Deanna et al. (2019), a bootstrap (BS) based on 1000 replications was also conducted to assess nodal support.

Morphological studies

The preserved specimens at the ABH herbarium and some additional preserved material from the online database of the MEXU herbarium from the UNAM-Mexico (datosabiertos.unam.mx) were also consulted (see Appendix 1). Geocoding of the Spanish localities of the plants was performed using a portable GPS device Garmin Montana 650t (Garmin, USA), with the geographic system UTM WGS84. The morphological features of the specimens were studied based on regional European and American Floras (Hawkes, 1972; Vargas et al., 2003; Sanz-Elorza & Sobrino, 2012; Valdés, 2012; Verloove, 2019), and specific research papers focused on the genus *Physalis* (Waterfall, 1958, 1967; D'Arcy, 1973, 1991; Sullivan, 2004; Ward, 2008; Landrum et al., 2013). The given detailed descriptions have been adapted from the above-mentioned literature and completed with observations of living plants and dried specimens at ABH and MEXU (see Appendix 3).

Results

Barcoding data

The first three BLAST top matches are reported for both unknown *Physalis* samples in Table 1. Our results revealed that the sequence ITS-Novelda had a clear correspondence with the taxonomic name *P. acutifolia* (Miers) Sandwith, since the two first matches showed a similar top score identity (99%). The second sequence (ITS-Elda) showed the same highest identity values with the three first top matches, which always corresponded to the species *P. angulata* L. Therefore, the two outputs provided a reliable taxonomic identification for both sequenced samples.

Table 1. BLAST identification of the two *Physalis* samples collected using ITS. Identification results were provided as the species name showing the top nearest matches (minimum e-value). The first three maximum nearest matches with the species name, GenBank accession and identity scores are reported for each studied sample.

Sample	Species	GenBank	Identity
ITS-Novelda	*Physalis acutifolia*	KY968916	99%
	Physalis acutifolia	AY665876	98%
	Physalis crassifolia	AY665889	97%
ITS-Elda	*Physalis angulata*	MH050300	100%
	Physalis angulata	MH763725	100%
	Physalis angulata	AY665875	100%

ITS showed enough phylogenetic resolution to be useful for DNA barcoding for the genus *Physalis* (Figure 1). An initial NJ tree showed that the resolution of the phylogenetic relationships among the species appeared in general well resolved, but the phylogenetic position of the sequences of certain species such as *P. angulata*, *P. minima* L., *P. peruviana* and *P. pubescens* L., showed noticeable discrepancies. The sequences correspondent to the species *P. angulata* and *P. peruviana* appeared divided in two well-separated groups, whereas the sequences for *P. pubescens* and *P. minima* were distributed in three different clades (Figure 1). Most of the sequences of *P. angulata* (GenBank codes: KX-KT), mostly from Feng et al. (2016) except for three sequences (GenBank codes: KT) from Wu et al. (unpublished work), appeared collapsed together with certain sequences of *P. minima* (GenBank codes: KX) and *P. pubescens* (GenBank codes: KP).

The sequences of the second clade of *P. angulata* (GenBank codes: MH-AY) were obtained by Whitson & Manos (2005), Deanna et al. (2019) and Zhu (unpublished work). Similarly, the clustering of the sequences of *P. peruviana*, *P. minima* and *P. pubescens* seems to be also related to the origin of these sequences. Based on these observations, the phylogenetic discrepancies observed in the tree might be more related to the publication of origin of the sequences than to their taxonomic identity. Therefore, only the sequences of these four species that met the following criteria were kept: sequences of a species that clustered together were originated from at least two different publications, and at least one of those sequences came from publications based on taxonomic revisions of the genus whose plant material was mainly collected from its original distribution (mainly Whitson & Manos, 2005, and Deanna et al., 2019). As a consequence, sequences of *P. angulata* from the first clade (GenBank codes: KX-KT) were removed, as well as six sequences from *P. minima*, 19 from *P. pubescens* and one from *P. peruviana*. Other species also showed phylogenetic discrepancies (e.g. *P. hederifolia* A. Gray, *P. cinerascens* Hitchc and *P. virginiana* Mill.). However, no sequence was removed since all these sequences came from plant material collected from its original distribution.

As a consequence, a second NJ tree was conducted with a total of 92 ITS sequences, which also showed a notable phylogenetic resolution (Figure 2). Based on this second tree, the here obtained sequences of the two unknown *Physalis* taxa were, hence, analyzed in order to find out their likely taxonomic identity based on their phylogenetic position with the closest *Physalis* taxa. The first unknown sequence (ITS-Novelda, GenBank code MT229084) clustered with two sequences of the species *P. acutifolia*, all of which conformed a well-supported monophyletic group (99% BS). The second unknown sequence (ITS-Elda, GenBank code: MT229083) grouped within the monophyletic clade of the sequences of the species *P. angulata*, with a high BS support (79% BS). Therefore, these two unknown sequences were identified by DNA barcoding as *P. acutifolia* and *P. angulata*, respectively.

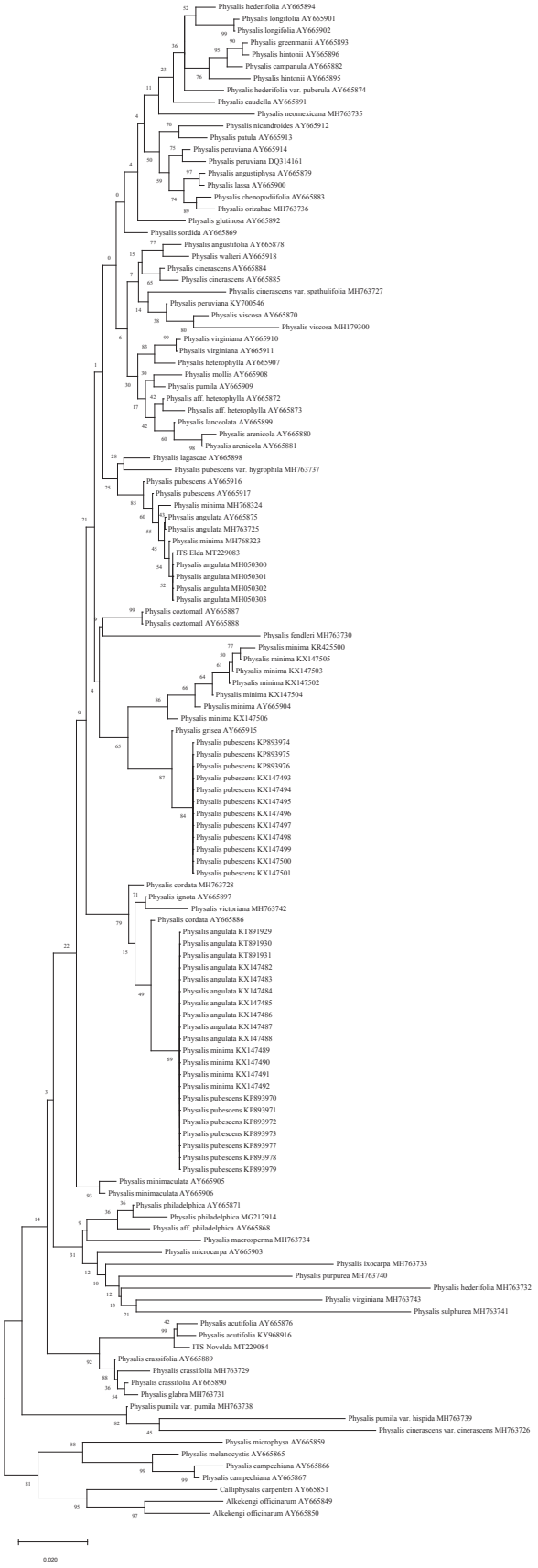

Figure 1. Neighbour-Joining (NJ) tree of all the internal transcribed spacer (ITS) sequences retrieved from GenBank. Numbers in branches indicate bootstrap support (BS) based on 1000 replications.

Figure 2. Neighbour-Joining (NJ) tree of the internal transcribed spacer (ITS) sequences after removing the misidentified sequences. Numbers in branches indicate bootstrap support (BS) based on 1000 replications.

Morphological data

A comparative study of the morphological characters of the mentioned *Physalis* species in the Iberian Peninsula and other western European Mediterranean areas was done, including the obtained morphological data of the collected plant material (Table 2). Similarly to barcoding results, the two collected specimens entirely matched with the vegetative and reproductive features of the annual species *P. acutifolia* and *P. angulata*, respectively (Table 2). Firstly, one of the specimens was easily identified as *P. acutifolia* by pubescent indumentum (simple hairs), lanceolate or linear-lanceolate leaves, white color and rotate corolla, size of the fruiting calyx (13-16 × 12-15 mm) and length of the fruiting pedicel (20-28 mm). This taxon is, in general, well differentiated against the remaining studied annual *Physalis* taxa, mainly based on the smaller size of the leaves, the larger length of the flowering and fruiting pedicel, the color and shape of the flower and the smaller length of the fruiting calyx (Table 2, Appendixes 3 and 4).
Secondly, the morphological study also confirms the identification of the second collected plant material with the species *P. angulata* thanks to the glabrous or subglabrous indumentum, the ovate or ovate-oblong leaves, the campanulate yellowish flowers without observable spots, the length of the flowering and fruiting pedicel (8-12 and 16-20 mm, respectively) and the size of the fruiting calyx (28-34 × 22-28 mm). Although this taxon appears more closely related to other alien annual species as *P. ixocarpa* and *P. philadelphica*, the species *P. angulata* is notably differentiated by its longer length of the flowering and fruiting pedicels and its smaller size of the fruiting calyx (Table 2, Appendixes 3 and 4).

Discussion

Although the impact of the alien plants in nature is often unpredictable, their proper identification will lay a solid foundation for further actions (Xu et al., 2018). Pyšek et al. (2013) already indicated the requirement of the use of genetic tools to identify alien taxa, in combination with traditional taxonomy based on the ability to assess morphological samples. Most of the recent identifications of alien flora in the Iberian Peninsula (Spain) were mostly based on morphological data (e.g. Verloove & Sánchez-Gullón, 2010; Gómez-Serrano & Laguna, 2011; Senar et al., 2017; Guillot & Laguna, 2019; among others), and sometimes, the morphological features could be somehow different from those from the wild areas. Therefore, the identification could take a long time since the plant was initially discovered, since no previous morphological references or checklists are always available (Pyšek et al., 2013).

Table 2. Diagnostic morphological characters between the here collected plant material of *Physalis acutifolia* (ABH 80489) and *P. angulata* (ABH 80490), and the bibliographic data for the annual species mentioned in the Western Mediterranean area (*P. acutifolia*, *P. angulata*, *P. philadelphica*, *P. ixocarpa*, *P. pubescens* and *P. nicandroides*).

	***P. acutifolia* (ABH 80489)**	***P. angulata* (ABH 80490)**	***P. acutifolia****	***P. angulata****	***P. philadelphica****	***P. ixocarpa****	***P. pubescens****	***P. nicandroides****
Indument	pubescent, short simple appressed hairs	glabrous, sparsely hairy (mainly in the apex fruiting lobes)	sparsely pubescent, with simple appressed hairs	glabrous or glabrescent (with simple appressed hairs)	subglabrous to sparsely hairy, with simple appressed hairs	subglabrous, with short appressed hairs	villous to glabrous, with glandular hairs	glandular-tomentose, with both simple and glandular hairs
Leaves								
Size	15-42 × 8-14	49-77 × 26-45	20-125 × 10-15	25-100(140) × 10-80	20-70 × 20-40	20-70 × 10-40	15-90 × (12)20-80	(30)60-120 × (25)50-160
Shape	lanceolate	ovate, ovate-oblong	ovate-lanceolate to linear-lanceolate	ovate, elliptic to linear-lanceolate	ovate to ovate-lanceolate	ovate to ovate-lanceolate	broadly ovate to orbicular	ovate to cordate
Margin	sinuate-dentate	sinuate-dentate	irregularly and coarsely dentate	deeply and coarsely irregularly dentate	dentate to entire	entire to dentate or sinuate-dentate	coarsely dentate or entire	entire to serrate
Petiole L	5-25	14-27	15-70	(20)40-80	(10)20-50	(10)20-50	20-70	10-90
Flowers								
Pedicel L	20-27	8-12	12-30	5-17(25)	3-7(12)	3-5	3-9	2-3
Calyx L	3.0-4.0	3.5-4.0	3.0-5.0	(2.0)3.0-7.0	5.0-10.0	4.0-6.0(10)	3.0-10.0	5.0-8.0
Calyx lobes L	1.0-2.0	1.5-2.0	1.0-2.0	1.0-3.0	2.0-4.0	2.0-4.0	1.0-4.0	3.0-5.0
Corolla D	20-23	7-12	(10)15-23	4-12	8-22	5-6	7-15	4-6(8)
Color	white	yellow	yellowish, white	yellow	yellow	yellow	yellow	whitish-cream
Presence of spots	yes, yellowish	not observable	yes, yellowish or orange	no or faintly tinged purple	yes, blue-tinged or purplish	yes, bluish-tinged	yes, dark-maculate	yes, greenish
Shape	rotate	campanulate	rotate	campanulate	campanulate	campanulate	campanulate	campanulate
Anthers								
Length	2.0-2.5	1.5-2.0	3.0-4.0	1.0-3.0	3.0-4.0	1.2-1.5(3)	1.0-3.0	1.4-2.0
Color	blue	yellow and blue	yellow with a blue or a blue-green tinge	blue or blue-tinged	blue	blue or yellowish with blue margins	bluish or violet	blue or blue-greenish
Stamens twisted	no	no	no	no	yes, after dehiscence	yes, after dehiscence	no	no
Fruit								
Pedicel L	20-28	16-20	25-60	(7)10-30	3-8(11)	3-8	5-15	5-25
Nº angles	10-angled	10-angled	10-angled	10-angled	10-angled	10-angled	5-angled	5-angled
Calyx size	13-16 × 12-15	28-34 × 22-28	12-25 × 10-20	10-40 × 7-30	20-30(50) × 20	20-30(50) × 20	18-40 × 17-38	35-45 × 25-40
Size	5-9	13-14	6-13	5-13	40-60	up to 10	7-18	10-22

* Data taken from Waterfall (1958, 1967), Sullivan (2004), Landrum et al. (2013) and Sanz-Elorza & Sobrino (2012). Range of values of the quantitative data are exposed in mm. For each range, exceptional measurements are given in brackets. Abbreviations: L, length; D, diameter.

Although the molecular data could fail in the taxonomic identification, it should still be considered as a useful approach since the results would at least reveal the closest taxonomic group to which the unknown alien taxa would belong. Our results support the use of DNA barcoding as an efficient tool for identifying allochthonous plant species, especially for those unmentioned taxa within a specific geographical area. Based on the integrative taxonomy (Dayrat, 2005), the first-step molecular study should be combined with *a posteriori* comparative morphological analyses, which would support the final identification of the unknown taxa, especially about those complex taxonomic groups. Regarding the morphological study, the Spanish sample of *P. acutifolia* shows morphological features (e.g. color and shape of the corolla, indumentum of the plant, morphology of the leaves) that fully fit the diagnostic characteristics of this species, though the leaves, petioles, anthers and fruiting calyx pedicel display smaller measures (Table 1). These weak morphological variations are probably due to the particular ecological and climatic conditions of the Spanish location. In the case of *P. angulata*, the Spanish specimen shows morphological features that entirely match with the typical diagnostic features of this species, though the length of the leaf petiole is smaller and the anther color is yellow and blue (Table 1). Waterfall (1958, 1967) reported the notable morphological plasticity of *P. angulata*, and recognized certain taxonomic varieties (e.g. *P. angulata* var. *lanceifolia* (Nees) Waterf. and *P. angulata* var. *pendula* (Rydb.) Waterf.) on the basis of narrower leaves (lanceolate and linear-lanceolate), among other morphological features. Nonetheless, Vargas et al. (2003), Sullivan (2004) and Landrum et al. (2013), and conversely to Ward (2008), did not recognize them, since they stated the existence of a continuous morphological intermixture without a clear geographical or ecological distinction. Moreover, some vegetative specimens of *P. angulata*, characterized by lanceolate leaves, could be easily misidentified with *P. acutifolia*, as Sullivan (2004) and Vargas et al. (2003) reported for field and herbarium material. Our data revealed that DNA barcoding tools would be quite useful for their proper taxonomic identification, since the phylogenetic position of *P. acutifolia* and *P. angulata* was quite distant (Figure 2). Consequently, the combination of molecular and morphological data has become a useful mechanism for determining the taxonomic identity of alien plants, as Pyšek et al. (2013) already suggested.

Nevertheless, the obtained molecular data should be initially treated carefully, since not all the sequences under the same taxonomic name would correspond to that same taxon. Our research has revealed the existence of different clusters of ITS sequences for certain *Physalis* species, as it happens with *P. angulata*. The sequenced samples of a particular species from different publications did not always cluster together, and their phylogenetic position seemed to be more related to the original publication than to its taxonomic identity, suggesting that the discrepancies for these species should be caused by confusions in the taxonomic identification from the original publication. In fact, five of the 13 different origins of the sequences available in NCBI database showed sequences misidentified, even in certain articles whose main objective was to use DNA barcoding to identify species. In addition, the lack of plant material collected under custody in official herbaria might also increase the level of misunderstanding since no plant material can be revised at a later date. Nilsson et al. (2006) already reported that the public DNA databases are not always sufficiently complete for the search entries, and even the taxonomic identification of the annotated entries might be misidentified. In the case of *Physalis*, some relevant discrepancies can be found when comparing the information available in the NCBI GenBank dataset with the final publication of these sequences, like orthographical errors and, even more crucially, taxonomic changes for the species names. Therefore, caution must be taken when directly using public databases to identify species by DNA barcoding, and therefore, the use of reliable sequences that have been produced in taxonomic studies is strongly encouraged. Our two studied samples of *P. acutifolia* and *P. angulata* were properly matched to those previously sequenced samples from native geographical areas, which have been also included in taxonomic treatments of the genus (Whitson & Manos, 2005; Deanna et al., 2019). Therefore, the morphological characterized samples of *P. acutifolia* and *P. angulata* used in this study as comparison for genetic analyses were correctly assigned by ITS, therefore confirming the reliability of DNA barcoding to identify these alien plant samples.

Conclusions

The integrative taxonomic approach has revealed to be a powerful tool to identify alien flora. This study highlights the usefulness of the combination of molecular plus morphological data to infer the taxonomic identity of allochthonous species. The exclusive use of traditional morphological approaches could be partial and not conclusive by themselves due to the lack of the main diagnostic morphological characters, among other reasons. However, caution must be taken when using sequences from public databases, since misidentified sequences can lead to taxonomic misidentifications of unknown sequences, and hence, morphological studies are also crucial to avoid potential mistakes. The complex taxonomy of the genus *Physalis* reveals the high similarity between certain species (see Sullivan, 2004), and the integrative taxonomy would become the adequate approach for determining the taxonomic identity of their species, mainly out of their natural distribution area. Our molecular

and morphological study reports the first European record for *P. acutifolia* and the first Spanish record for *P. angulata*, which also corresponds to the second mention for a Western Mediterranean European country.

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Appendix 1

Selected specimens examined

Physalis acutifolia (Miers) Sandwith

Hs, Alicante: Novelda, próximo al río Vinalopó, margen derecha, matorral halo-nitrófilo de *Atriplex halimus*, 38° 25’ 40.9’’N, 0° 48’ 33.3’’W, 305 m a.s.l., 27-10-2018, A. Juan AJ260 (ABH 80489). Mx, Baja California: Llano de Caquihui, Sierra de la Giganta west of Los Dolores, 17-11-1959, Ira L. Wiggins 15503 (MEXU 107944). Mx, Campeche: Palizada, 25/28-07-1939, E. Matuda 3867 (MEXU 83815). Mx, Sinaloa: Concordia, Panuco, Santa Lucia, 1000 m a.s.l., Ing. Jesús González Ortega 5933 (MEXU 29051); Salvador Alvarado, 40 m a.s.l., 29-09-1991, G. Bojórquez Bojórquez 822. Mx, Sonora: Rancho Los Pescados, drainage S to Rio Aros, 29.483º, -108.983º, 780 m a.s.l., Elaine Joyal 1736, M. Silva C. & M. E. Silda D. (MEXU 579147); Hermosillo, Calle 12 ± 10 km carr. a la costa, 40 m a.s.l., 27-08-1975, C. Rodríguez J. 1659 (MEXU 251939); Cerro Prieto, Microondas, 15 km al E de Navojoa, 80-320 m a.s.l., 3-10-1985, P. Tenorio L. 10199, C. Romero de T., J. Ignacio S. & Patricia Dávila (MEXU 678179).

Physalis angulata L.

Hs, Alicante: Elda, río Vinalopó, margen izquierdo del cauce, bajo dosel de *Tamarix gallica*, 38° 28’ 13.4’’N, 0° 48’ 16.1’’W, 370 m a.s.l., 02-09-2018, A. Juan AJ244 (ABH 80490). Mx, Guerrero: Chilpancingo, Rincón de La Vía, 17.2875°, -99.48194°, 21-3-1960, Hubert Kruze 217 (MEXU 1100037); Chilpancingo de los Bravos, Rincón de La Vía, 17.2875°, -99.48194°, 12-8-1962, Hubert Kruze 812 (MEXU 1100039). Mx, Jalisco: Carretera Cuotá Guadalajara-Lagos de Moreno, km 86, 1750 m a.s.l., 4-08-1995, Aarón Rodríguez 2717 (MEXU 1395791). Mx, Sinaloa: Culicán, Península de Lucenilla, ± 1 km de la entrada de la península, 7-9-1985, Faustino Hernandez & J.A. Gutierrez 284 (MEXU 1322838). Mx, Tabasco: Centro, a 100 m de desviación a Tamulté de las Sabanas, km 26 Carretera Villahermosa-Frontera, 18-1-1995, M.A. Guadarrama O. & M.A. Magaña A. 95-1-3 (MEXU 1013789).

Appendix 2

NCBI GenBank numbers of the internal transcribed spacer (ITS) sequences used in the article. The country of origin from the plant material is indicated when available in the original reference. The taxon name and authors were written according to IPNI (www.ipni.org).

Species	GenBank numbers	Country	Reference	Observations
Alkekengi officinarum Moench	AY665849-AY665850	Cultivated	Whitson & Manos (2005)	as *P. alkekengi* in the original paper
Calliphysalis carpenteri (Riddle) Whitson	AY665851	USA	Whitson & Manos (2005)	as *P. carpenteri* in the original paper
Physalis acutifolia (Miers) Sandwith	AY665876	USA	Whitson & Manos (2005)	
	KY968916	China	Xu et al. (2018)	
	MT229084	Spain	This study	Novelda
Physalis angulata L.	AY665875	USA	Whitson & Manos (2005)	
	KT891929-KT891931	Unknown	Wu et al. (unpublished)	
	KX147482-147488	China	Feng et al. (2016)	
	MH050300-MH050303	China	Zhu (unpublished)	
	MH763725	Argentina	Deanna et al. (2019)	
	MT229083	Spain	This study	Elda
Physalis angustifolia Nutt.	AY665878	USA	Whitson & Manos (2005)	
Physalis angustiphysa Waterf.	AY665879	Mexico	Whitson & Manos (2005)	
Physalis arenicola Kearney	AY665880-AY665881	USA	Whitson & Manos (2005)	
Physalis campanula Standl. & Steyerm.	AY665882	Mexico	Whitson & Manos (2005)	as *P. campanulata* in GenBank
Physalis campechiana L.	AY665866-AY665867	Mexico	Whitson & Manos (2005)	as *P. arborescens* in the original paper

Species	GenBank numbers	Country	Reference	Observations
Physalis caudella Standl.	AY665891	Mexico	Whitson & Manos (2005)	
Physalis chenopodii-folia Lam.	AY665883	Cultivated	Whitson & Manos (2005)	as *P. chenipodifolia* in the original paper
Physalis cinerascens Hitchc.	AY665884-AY665885	USA	Whitson & Manos (2005)	
Physalis cinerascens var. *cinerascens*	MH763726	USA	Deanna et al. (2019)	
Physalis cinerascens var. *spathulifolia* (Torr.) J.R. Sullivan	MH763727	USA	Deanna et al. (2019)	
Physalis cordata Mill.	AY665886	USA	Whitson & Manos (2005)	
	MH763728	Peru	Deanna et al. (2019)	
Physalis coztomatl Moc. & Sessé ex Dunal	AY665887-AY665888	Mexico	Whitson & Manos (2005)	
Physalis crassifolia Benth.	AY665889	USA	Whitson & Manos (2005)	
	AY665890	Mexico	Whitson & Manos (2005)	
	MH763729	USA	Deanna et al. (2019)	
Physalis fendleri A.Gray	MH763730	USA	Deanna et al. (2019)	
Physalis glabra Benth.	MH763731	Mexico	Deanna et al. (2019)	
Physalis glutinosa Schltdl.	AY665892	Mexico	Whitson & Manos (2005)	
Physalis greenmanii Waterf.	AY665893	Mexico	Whitson & Manos (2005)	
Physalis grisea (Waterf.) M.Martínez	AY665915	Cultivated	Whitson & Manos (2005)	as *P. pruinosa* in GenBank
Physalis hederifolia A.Gray	AY665894	USA	Whitson & Manos (2005)	as *P. hederaefolia* in the original paper
	MH763732	USA	Deanna et al. (2019)	
Physalis hederifolia var. *puberula* A.Gray	AY665874	Mexico	Whitson & Manos (2005)	as *P. hederaefolia* var. *puberula* in the original paper
Physalis heterophylla Nees	AY665907	USA	Whitson & Manos (2005)	
Physalis aff. heterophylla	AY665872-AY665873	USA	Whitson & Manos (2005)	
Physalis hintonii Waterf.	AY665895-AY665896	Mexico	Whitson & Manos (2005)	
Physalis ignota Britton	AY665897	Mexico	Whitson & Manos (2005)	
Physalis ixocarpa Brot. ex Hornem.	MH763733	USA	Deanna et al. (2019)	
Physalis lagascae Roem. & Schult.	AY665898	Mexico	Whitson & Manos (2005)	
Physalis lanceolata Michx.	AY665899	USA	Whitson & Manos (2005)	
Physalis lassa Standl. & Steyerm.	AY665900	Mexico	Whitson & Manos (2005)	
Physalis longifolia Nutt.	AY665901-AY665902	USA	Whitson & Manos (2005)	
Physalis macrosperma Pyne, E.L.Brdiges & Orzell	MH763734	USA	Deanna et al. (2019)	
Physalis melanocystis Bitter	AY665865	Mexico	Whitson & Manos (2005)	
Physalis microcarpa Urb. & Ekman	AY665903	Mexico	Whitson & Manos (2005)	

Species	GenBank numbers	Country	Reference	Observations
Physalis microphysa A.Gray	AY665859	Mexico	Whitson & Manos (2005)	
Physalis minima L.	AY665904	Thailand	Whitson & Manos (2005)	
	KR425500	India?	Santhosh Kumar et al. (unpublished)	
	KX147502-KX147506	China	Feng et al. (2016)	
	KX147489-KX147492	China	Feng et al. (2016)	as *P. angulata* var. *villosa* in the original paper
	MH768323-MH768324	China	Li et al. (2018)	
Physalis minimaculata Waterf.	AY665905-AY665906	Mexico	Whitson & Manos (2005)	
Physalis mollis Nutt.	AY665908	USA	Whitson & Manos (2005)	
Physalis neomexicana Rydb.	MH763735	USA	Deanna et al. (2019)	
Physalis nicandroides Schltdl.	AY665912	Mexico	Whitson & Manos (2005)	
Physalis orizabae Dunal	MH763736	Mexico	Deanna et al. (2019)	
Physalis patula Mill.	AY665913	Mexico	Whitson & Manos (2005)	
Physalis peruviana L.	AY665914	Ecuador	Whitson & Manos (2005)	
	DQ314161	Ecuador	Smith & Baum (2006)	
	KY700546	Unknown	Moorhouse-Gann et al. (unpublished)	
Physalis philadelphica Lam.	AY665871	Cultivated	Whitson & Manos (2005)	
	MG217914	Canada	Kuzmina et al. (2017)	
Physalis aff. philadelphica	AY665868	Cultivated	Whitson & Manos (2005)	
Physalis pubescens L.	AY665916-AY665917	Costa Rica	Whitson & Manos (2005)	
	KP893970-KP893979	Unknown	Yanan et al. (unpublished)	
	KX147493-KX147501	China	Feng et al. (2016)	
Physalis pubescens var. *hygrophila* (Mart.) Dunal	MH763737	Argentina	Deanna et al. (2019)	as *P. angulata* var. *higrophyla* in the original paper
Physalis pumila Nutt.	AY665909	USA	Whitson & Manos (2005)	
Physalis pumila var. *pumila*	MH763738	USA	Deanna et al. (2019)	
Physalis pumila var. *hispida* (Waterf.) J.R. Sullivan	MH763739	USA	Deanna et al. (2019)	
Physalis purpurea Wiggins	MH763740	Bolivia	Deanna et al. (2019)	
Physalis sordida Fernald	AY665869	Mexico	Whitson & Manos (2005)	
Physalis sulphurea (Fernald) Waterf.	MH763741	Mexico	Deanna et al. (2019)	
Physalis victoriana J.M. Toledo	MH763742	Argentina	Deanna et al. (2019)	
Physalis virginiana Mill.	AY665910-AY665911	USA	Whitson & Manos (2005)	
	MH763743	USA	Deanna et al. (2019)	
Physalis viscosa L.	AY665870	Cultivated	Whitson & Manos (2005)	
	MH179300	Argentina	Ibañez et al. (2019)	
Physalis walteri Nutt.	AY665918	USA	Whitson & Manos (2005)	

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Appendix 3

Morphological description and distribution of the new *Physalis* alien plants

Physalis acutifolia (Miers) Sandwith, Kew Bull. 14: 232 (1960)

Annual herb, up to 50 cm, sparsely pubescent with short non-glandular antrorse hairs on stems, pedicels, leaf edges, veins and lobe margins of the fruiting calyx; subglabrous on the base of the stem. Leaves 20-125 × 10-15 mm, alternate, ovate-lanceolate to linear-lanceolate, base acute-attenuate often obliquely, apex acuminate, margins sinuate and irregularly and coarsely dentate; petiole 15-70 mm. Inflorescence reduced to a unique axillary flower. Flowers actinomorphic, bisexual, rotate, ebracteate; pedicels 12-30 mm. Calyx 3-5 mm long, the lobes 1-2 mm long, about as long as the tube, acuminate. Corolla white to yellowish-white, with a yellowish or orange darkened centre, margins ciliate, (10)15-23 mm diameter, 10-15 mm long. Anthers 3-4 mm long, blue-green or yellow with a blue tinge, not coiled after dehiscence; filaments 4-5 mm long, free. Stigma capitate; style 7-9 mm long. Fruiting calyx, 12-25 × 10-20 mm, green with purplish-blackish nerves, lobes acute or acuminate clearly pubescent on their edges; fruiting pedicel 25-60 mm; fruit 6-13 mm diameter. Seeds lenticular 2-2.2 mm wide.

The plant was discovered on semi-arid open sunny places in the surroundings of the river Vinalopó close to Novelda city (Alicante province), at 305 m a.s.l. (38° 25’ 40.9’’N, 0° 48’ 33.3’’W). It occurs under nitro-halophilous plant communities dominated by the shrub *Atriplex halimus* L., together with other species such as *Lobularia maritima* (L.) Desv. subsp. *maritima*, *Phagnalon saxatile* (L.) Cass. and *Cirsium arvense* (L.) Scop. The area is located in the Thermomediterranean xeric oceanic stage (Rivas-Martínez et al., 2004), with an average annual temperature of 17 °C and an average annual rainfall of 355 mm (www.climate-data.org). According to our current observations, *Physalis acutifolia* should be considered as a casual alien, whose origin is still not clear. Recently, Rebbas (2018) has proposed the agricultural uses as the potential source of this species in Algeria. Further field surveys would be needed to identify the potential provenance of this species and also to seek new localities.

Physalis angulata L., Sp. Pl. 1: 183 (1753)

Annual herb, up to 30 cm, glabrous or glabrescent with small non-glandular hairs, mainly on the young parts and the edge of the fruiting lobes. Leaves 25-100(140) × 10-80 mm, alternate, ovate, elliptic to linear-lanceolate, base rounded to attenuate, apex acute to acuminate, margin deeply and coarsely irregularly dentate; petiole (20)40-80 mm. Inflorescence reduced to a unique axillary flower. Flowers actinomorphic, bisexual, campanulate, ebracteate; pedicels 5-17(25) mm. Calyx (2)3-7 mm long, lobes 1-3 mm long, tube equal or longer than the lobes. Corolla yellow, with purplish smudges but sometimes not observable, 4-12 mm diameter. Anthers 1-3 mm long, blue or blue-tinged, not coiled after dehiscence; filaments 3-5 mm long, free. Stigma capitate. Fruiting calyx 10-40 × 7-30 mm, green, lobes acute; fruiting pedicel (7)10-30 mm; fruit 5-13 mm diameter. Seeds lenticular 2-2.2 mm wide.

The plant was discovered on the stream margins of the river Vinalopó (Elda, Alicante province), at 370 m a.s.l. (38° 28’ 13.4’’N 0° 48’ 16.1’’W) growing on sandy soils covered by monospecific grasses dominated by the species *Stenotaphrum secundatum* (Walt.) Kuntze, which is also an allochthonous plant species, with an invasive behavior along the margins of the river Vinalopó. Frequently occurring taxa close to this area are: *Tamarix gallica* L., *Physalis peruviana* L., *Brassica oleracea* L., *Mesembryanthemum crystallinum* L., *Amaranthus muricatus* (Moq.) Hieron., *Glebionis coronaria* (L.) Cass. ex Spach and *Portulaca oleracea* L. The area is located in the Mesomediterranean dry oceanic stage (Rivas-Martínez et al., 2004), with an average annual temperature of 16.1 °C and an average annual rainfall of 393 mm (www.climate-data.org). According to Pyšek et al. (2004), and at this stage of knowledge, the presence of this alien plant must be considered as a casual alien, whose origin is not clear. Recently, this species was also mentioned as a casual alien growing close to a lake shore in south Italy (Musarella et al., 2020), with a similar ecology to the Spanish specimen (riverbank shore). However, this plant has been mostly cited from waste grounds, fields and arable crops from Turkey, Anatolia, Libya and Belgium (Gönen et al., 2000; Ozaslan et al., 2017; Mahklouf, 2019; Verloove, 2019), and hence it might be introduced with manure from cultivated fields. Further field research would be needed to identify the potential origin of this species close to aquatic habitats. In addition, a downstream search for the likely presence of new Spanish populations would be needed, since this species shows a high seed germination percentage under a wide range of environmental conditions, which would suggest a further invasion potential (Ozaslan et al., 2017).

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Appendix 4

Dichotomous key

Dichotomous key for the identification of the mentioned *Physalis* in the Western Mediterranean area, excepting for the species *Physalis alkekengi*, which is here recognized as *Alkekengi officinarum* based on molecular evidence and morphological characteristics.

1. Perennial plants 2
– Annual plants 4
2. Leaves 50-100 × 40-70 mm; flowering pedicel 6-8 mm long; anthers bluish; fruiting pedicels 7-11 mm long *Physalis peruviana* L.
– Leaves 20-70 × 10-50 mm; flowering pedicels 9-20 mm long; anthers yellowish; fruiting pedicels 13-30 mm long 3
3. Plants with pubescence hispid with simple and short retrorse hairs; corolla 15-25 mm diameter, with purple-brown spots in throat *Physalis virginiana* Mill.
– Plants covered by stellate and glandular hairs; corolla 8-16 mm diameter, with greenish-yellow spots in throat *Physalis viscosa* L.
4. Plant pubescent, glandular-tomentose or villous, with glandular hairs 5
– Plant glabrous to subglabrous, or pubescent with non-glandular simple hairs 6
5. Large leaves up to 120 × 160 mm; corolla, 4-6(8) mm diameter, whitish-cream with greenish spots in throat *Physalis nicandroides* Schltdl.
– Leaves smaller up to 90 × 80 mm; corolla, 7-15 mm diameter, yellow often with dark spots in throat *Physalis pubescens* L.
6. Plant clearly pubescent; corolla white with yellowish-orange spots in throat *Physalis acutifolia* (Miers.) Sandwith
– Plant mostly glabrous to subglabrous; corolla yellow often with purplish or bluish spots in throat 7
7. Throat of corolla unspotted or with faint dark spots; flowering pedicel 5-17(25) mm long, and fruiting pedicel 10-30 mm long *Physalis angulata* L.
– Throat of corolla with distinct dark spots; flowering peduncle 3-7(12) mm, and fruiting pedicel up to 11 mm 8
8. Corolla 8-22 mm in diameter; fruit size 40-60 mm *Physalis philadelphica* Lam.
– Corolla 5-6 mm in diameter; fruit size up to 10 mm *Physalis ixocarpa* Brot.