

## MOLECULAR PHYLOGENY INDICATES POLYPHYLY IN *RAPHANUS* L. (BRASSICACEAE)

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We evaluated the systematic relationship of the two sections of *Raphanus* (Brassicaceae) on the basis of morphological and ITS data. We obtained strong support that *Raphanus* is a polyphyletic group embedded in the *Oleracea* lineage of the tribe Brassiceae. Section *Raphanis*, which includes *Raphanus raphanistrum*, *R. pugioniformis* and *R. sativus*, is a strongly supported monophyletic lineage. Section *Hesperidopsis* is embedded in a different lineage together with *Brassica deflexa* and *B. aucheri*. We propose to reinstate the genus *Quidproquo* in place of *Raphanus* sect. *Hesperidopsis* as reflected by both morphological data and ITS phylogeny.

*Keywords.* Brassiceae, internal transcribed spacer, morphology, *Quidproquo*, radish, silique.

### INTRODUCTION

The genus *Raphanus* L. [Brassicaceae, lineage 2, tribe Brassiceae (Franzke *et al.*, 2011)] is characterised, like all other members of the tribe, by heteroarthrocarpous siliques, transversally divided into two differently structured segments: a proximal, dehiscent (valvar) and a distal, indehiscent (stylar) segment (Avino *et al.*, 2012). Unlike other members of the tribe, the heteroarthrocarpous silique of *Raphanus* is modified into a completely indehiscent, unilocular, cylindrical, thick-walled fruit, a result of the reduction of the valvar segment, and the fully developed, multi-seeded stylar segment (Schulz, 1919; Pistrick, 1987).

In a taxonomic synopsis of *Raphanus*, Schulz (1919) recognised two sections:

- 1 Section *Raphanis* DC., which is characterised by erect sepals, relatively long, clawed petals (1.6–2.6 cm), erect-spreading siliques and round to ovoid seeds. This section includes the polymorphic and subcosmopolitan wild radish, *Raphanus raphanistrum* L., under which at least two subspecies can be counted (Warwick *et al.*, 2006b), the highly variable cultivated radish, *R. sativus* L., and the Levant-endemic

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*R. pugioniformis* Boiss., often classified as a variety of *R. raphanistrum* (Warwick *et al.*, 2006b).

- 2 Section *Hesperidopsis* Boiss., which is characterised by patent sepals, short petals (1 cm), pendulous siliques and oblong seeds. This section is monotypic, consisting of *Raphanus confusus* (Greuter & Burdet) Al-Shehbaz & Warwick, formerly known by the illegitimate name *R. aucheri* O.E.Schulz (Al-Shehbaz & Warwick, 1997 and references therein) which is geographically restricted to northern Israel, southern Syria and Lebanon (Baillargeon, 1985a).

For the past three decades there has been much controversy over the systematic position of section *Hesperidopsis*. Greuter and Burdet (in Greuter & Raus, 1983) considered it to be a separate genus and created the binomen *Quidproquo confusum* Greuter & Burdet for *Raphanus aucheri*. Pistrick (1987), in a review of *Raphanus*, recognised Greuter and Burdet's approach, and also (Baillargeon, 1985a) supported the separation of genera, mentioning that the reduced and dysfunctional valvar segment in both genera may be a result of convergence or parallelism. By contrast, Al-Shehbaz & Warwick (1997) did not recognise the separate status of *Quidproquo*, arguing that differences between the proposed genera were too small to justify it. Moreover, they argued that Greuter and Burdet in Greuter & Raus (1983) had not described the differences between the putative genus *Quidproquo* and *Raphanus* at generic level. Here we refer to the species at its current systematic position, namely *Raphanus confusus*.

To understand the taxonomic position of *Raphanus confusus* in relation to other *Raphanus* species we created a synoptic matrix of morphological characters in an attempt to evaluate similarities and differences among target taxa. We further created a phylogenetic tree based on DNA sequences of the nuclear ribosomal 5.8S and its two internal transcribed spacer (ITS) regions, and compared the molecular phylogeny with morphological data. The ITS region is the most widely used DNA locus in crucifer systematics and taxonomy (Al-Shehbaz *et al.*, 2006).

## METHOD

Morphological evaluation of *Raphanus raphanistrum*, *R. pugioniformis*, *R. sativus* and *R. confusus* included various morphological traits traditionally used as taxonomic markers in this group, namely fruit, flower and seed morphology plus indumentum features. For the molecular phylogenetic study, we used four recognised *Raphanus* species, plus reference species from several Mediterranean clades of tribe Brassiceae (Warwick & Sauder, 2005); *Arabidopsis* (lineage 1, tribe Camelinae; Franzke *et al.*, 2011) was used as an outgroup (Table 1).

Leaf samples of *Raphanus* species were collected in their natural habitats in Israel, or species sequence information was gathered from an available database (<http://www.ncbi.nlm.nih.gov/>) (Table 1). Upon collection, leaf samples were placed in bags with silica gel and used for DNA isolation using an Invisorb Spin Plant Mini Kit (Stratec Molecular) following the manufacturer's procedure.

TABLE 1. Species and sequence data used for the current study. Accessions were either sequenced or sequences were downloaded from GenBank. Coordinates are given for accessions collected in Israel; accession numbers are given for sequences derived from the database

Species	Source	Coordinates (°)		Accession no.
		Longitude	Latitude	
<i>Raphanus pugioniformis</i>	Dishon, Galilee (DG)	35.514679	33.075361	
<i>Raphanus pugioniformis</i>	Hagilboa Reserve, Samaria (MS)	35.408635	32.504224	
<i>Raphanus pugioniformis</i>	Alma, Galilee (AG)	35.501804	33.056731	
<i>Raphanus pugioniformis</i>	Kadouri School, Galilee (KG)	35.407820	32.706386	
<i>Raphanus pugioniformis</i>	Kafr Kanna, Galilee (KKG)	35.352030	32.753968	
<i>Raphanus pugioniformis</i>	Lavi, Galilee (LG)	35.407800	32.775051	
<i>Raphanus pugioniformis</i>	Ein HaShofet, Manasseh Hills (ES)	35.104280	32.588984	
<i>Raphanus confusus</i>	Ein HaShofet, Manasseh Hills (ES)	35.104280	32.588984	
<i>Raphanus confusus</i>	Ein Fureir, Manasseh Hills (EFS)	35.105438	32.628171	
<i>Raphanus confusus</i>	Arbel, Galilee (AG)	35.499959	32.824788	
<i>Raphanus confusus</i>	Sepphoris, Galilee (ZG)	35.203521	32.433214	
<i>Raphanus raphanistrum</i>	Tel Aviv, Sharon plain (TS)	34.504311	32.173319	
<i>Raphanus raphanistrum</i>	Bet Dagan, Philistean plain (BP)	34.822927	31.991335	AY722479
<i>Raphanus raphanistrum</i>	GenBank (GB)			AY746462.1
<i>Raphanus sativus</i>	Diego (commercial cultivar)			FJ609733.1
<i>Raphanus sativus</i>	GenBank (GB)			DP249828.1
<i>Sinapis alba</i>	Bet Dagan, Philistean plain (BP)	34.822927	31.991335	U96268.1
<i>Sinapis alba</i>	GenBank (GB)			U43224.1
<i>Sinapis arvensis</i>	GenBank (GB)			
<i>Arabidopsis lyrata</i>	GenBank (GB)			
<i>Arabidopsis thaliana</i>	GenBank (GB)			
<i>Eruca sativa</i>	Yafit, Jordan Valley (YJ)	35.462923	32.050353	
<i>Eruca sativa</i>	Kingdom of Jordan, Northern Jordan Valley (JJ)	Unknown	Unknown	
<i>Eruca sativa</i>	Bet Shean, Jordan Valley (BJ)	35.497384	32.480805	
<i>Hirschfeldia incana</i>	Muqeible, Jezreel plain (MG1)	35.3021	32.5339	
<i>Hirschfeldia incana</i>	Muqeible, Jezreel plain (MG2)	35.3021	32.5339	

TABLE 1. (*Cont'd*)

Species	Source	Coordinates (°)		Accession no.
		Longitude	Latitude	
<i>Hirschfeldia incana</i>	Salfit, Samaria (SA)	35.2203	32.0918	AY722470.1
<i>Hirschfeldia incana</i>	GenBank (GB)			JN564039.1
<i>Brassica rapa</i>	GenBank (GB)			JQ085859.1
<i>Brassica napus</i>	GenBank (GB)			
<i>Brassica tournefortii</i>	Hadera, Sharon plain (HS)	34.872622	32.39029	
<i>Brassica tournefortii</i>	GenBank (GB)			AY722428.1
<i>Brassica aucheri</i>	GenBank (GB)			AY722413.1
<i>Brassica deflexa</i>	GenBank (GB)			GQ2680771
<i>Hemicrambe fruticulosa</i>	GenBank (GB)			AY722469.1
<i>Raffanaldia primuloides</i>	GenBank (GB)			AY722478.1

Nucleotide variation of the nuclear 5.8S rDNA and the ITS regions 1 and 2 was used to investigate phylogenetic relationships among the *Raphanus* species. PCR amplifications were performed with primer sets that were designed on the basis of known sequences of the 5.8S rDNA (CGACCGACGATATGACGAG for the forward, and the degenerate GAGAACGYTSRAWCATCACTCTC for the reverse, for which: Y- C or T; S- G or C; R- A or G; W- A or T). Clear amplification products were purified using the Qiaquick PCR purification kit (Qiagen) and sequenced at the Center of Genomic Technologies (The Hebrew University of Jerusalem, Israel). Others were ligated using the CloneJET PCR (Thermo Scientific), transformed to HIT competent cells (TM-JM109, RBC) and sequenced after plasmid purification (GeneJET plasmid miniprep, Thermo Scientific).

Alignment of the 5.8S and ITS sequences was performed by the MAFFT version 7 program using default parameters (<http://mafft.cbrc.jp/alignment/server/>). The phylogenetic tree was then reconstructed based on the maximum-likelihood (ML) framework using the PhyML software 3.0 (<http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html>) and the GTR model (Guindon *et al.*, 2010). The bootstrap consensus tree (Table 2) was inferred from 100 replicates (Felsenstein, 1985), and the percentage of replicate trees in which the associated clade clustered together in the bootstrap test was designated next to the branches (Felsenstein, 1985). The tree was graphically designed with the use of FigTree version 1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

## RESULTS AND DISCUSSION

In a matrix comparing 13 morphological traits in *Raphanus* species (Table 2), nine were shared by *Raphanus raphanistrum*, *R. pugioniformis* and *R. sativus*, but not by *R. confusus*. *Raphanus pugioniformis*, *R. raphanistrum* and *R. sativus* can be distinguished from *R. confusus* by their erect sepals, forming a tube-like calyx, markedly veined petals (Fig. 1), patent, attenuate fruit (thick in the middle, gradually becoming narrower), with trichomes concentrated and directed towards the apex of the fruit, and nearly spherical seeds (Fig. 2). By contrast, *Raphanus confusus* is distinguishable by patent sepals, unveined petals (Fig. 1), curled pedicels, pendulous siliques thickest towards the tip, silique trichomes concentrated at the base of the silique and directed towards the pedicel, and elliptical seeds (Fig. 2 and Table 2). An additional interesting unique feature in *Raphanus confusus* is the white spots on the adaxial leaf surface (Fig. 1).

The numerous distinct characteristics of *Raphanus confusus* support Greuter and Burdet in Greuter & Raus (1983) in questioning its systematic placement in *Raphanus*. Further support is provided by the phylogenetic tree based on ITS analysis which demonstrates the polyphyly of *Raphanus* sensu Schulz (1919) (Fig. 3). The analysis placed *Raphanus pugioniformis*, *R. raphanistrum* and *R. sativus* (section *Raphanis*) as a highly supported monophyletic group (bootstrap 100) which is sister

TABLE 2. Matrix of morphological traits in four *Raphanus* species according to Schulz (1919) and Post (1932)

Taxonomic section (Schulz, 1919)		<i>Raphanus</i>		
<i>Hesperidopsis</i>		<i>R. pugioniformis</i>		
<i>R. confusus</i>		<i>R. raphanistrum</i>		
<i>R. sativus</i> var. <i>radicula</i>				
Character				
Adaxial leaf surface colour	White-spotted green	Solid green	Solid green	Solid green
Pediceal at fruit maturity	Curled	Patent	Patent	Patent
Flower shape	Stellate	Pseudo-salverform	Pseudo-salverform	Pseudo-salverform
Petal venation	Inconspicuous	Conspicuous	Conspicuous	Conspicuous
Average petal length (cm)	1	1.8	1.8	1.8
Corolla colour	Sulphur yellow	Pink (rarely white)	White to pale yellow or to pale pink	Pink, rarely white
Trichome direction on fruit	Base-directed ('reflexed')	Apex-directed	Apex-directed	Apex-directed
Concentration of trichomes on fruit	Towards base	Towards apex	Towards apex	Towards apex
Seed shape	Oblong	Subspherical	Subspherical	Subspherical
Silique's widest region	Between middle and stylar tip	Between base and stylar tip	Between base and stylar tip	Between base and stylar tip
Stylar tip shape	Widely conical	Long-attenuate	Short-attenuate	Short-attenuate
Silique integrity when dry	Remains integral	Remains integral	Lomentaceous	Remains integral
Rest of valvar segment	Seedless	Seedless, rarely 1-seeded	Seedless, rarely 1-seeded	Seedless

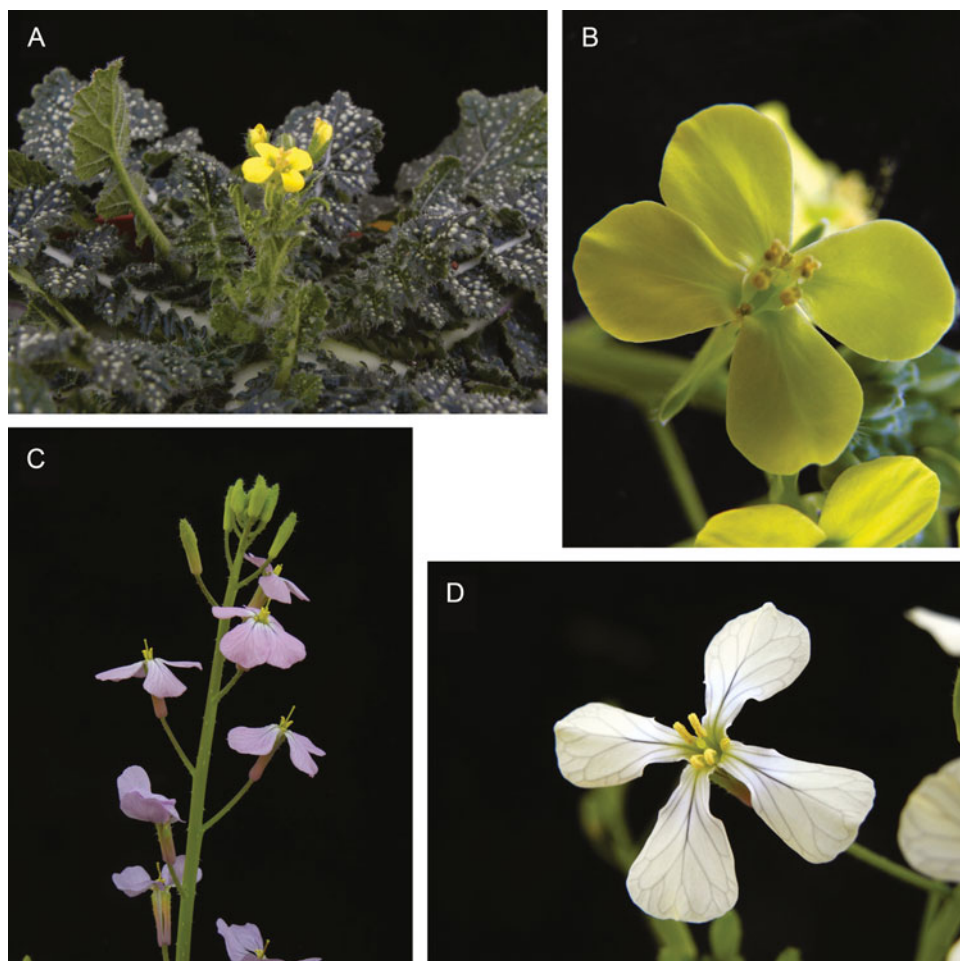


FIG. 1. *Raphanus* species. (A) *R. confusus* (= *Quidproquo confusum*) habit, showing white-spotted leaves; (B) *R. confusus* flower with patent sepals and solid-yellow petals; (C) *R. pugioniformis* inflorescence, showing erect sepals and dark-veined petals; (D) *R. raphanistrum* flower, showing dark-veined petals.

to *Hirschfeldia incana* and *Brassica tournefortii* (clade A, bootstrap 83); the monotypic section *Hesperidopsis* (*R. confusus*) is found in a highly supported monophyletic group with *Brassica deflexa* and *B. aucheri* in another lineage (clade B, bootstrap 84), which is sister to clade A (Fig. 3). The presumed close relationship between *Brassica aucheri* and *Raphanus confusus* is supported by the identical direction and location of trichomes on the silique of the two species, as can be observed in Fig. 2 and herbarium material [isotype: Iran, *Hausknecht* s.n., Apr. 1868 (JE!)]. This characteristic cannot be observed in *Brassica deflexa*, as its fruit is glabrous. A further morphological marker for clade B is the pendulous silique, which is a result of a curl (in *Raphanus confusus* and *Brassica aucheri*) or bend (in *B. deflexa*) of the pedicel. Moreover, in

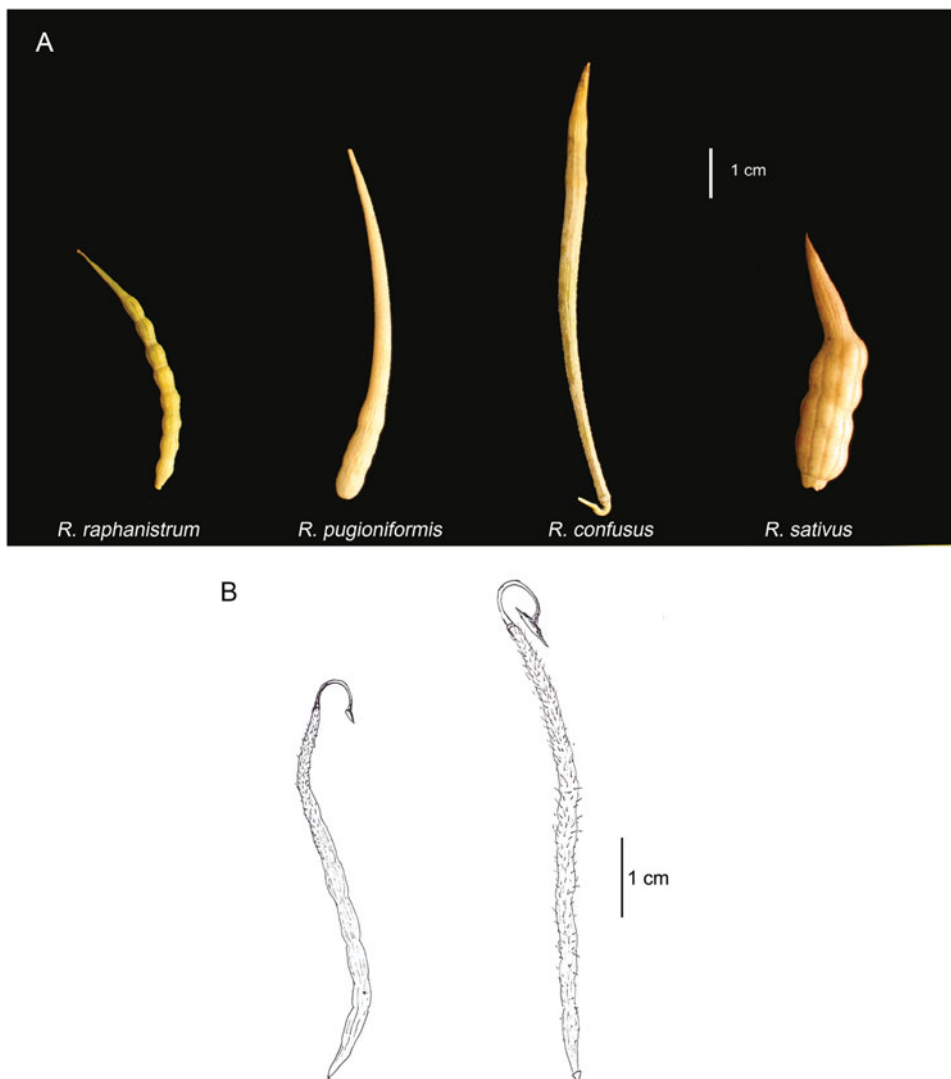


FIG. 2. Fruits of *Raphanus* species (A) and illustration of fruit of *R. confusus*, indicating the direction of trichomes on the fruit (B).

clade B, species have a strong resemblance in their flower form (stellate, due to patent sepals and petal) and colour (yellow).

Our ITS results deviate from the chloroplast-based phylogenies proposed by Warwick & Hall (2009) and Arias & Pires (2012) and clearly show the need for more elaborate studies elucidating the phylogenetic position of *Raphanus confusus*. Both chloroplast-based phylogenetic trees relate *Raphanus raphanistrum* to the monophyletic *Oleracea* clade, which also includes *Eruca* spp., *Brassica deflexa*, *B. napus* and *B. rapa*, while *Hirschfeldia incana* and *Brassica tournefortii*, *Raffenaldia primuloides*



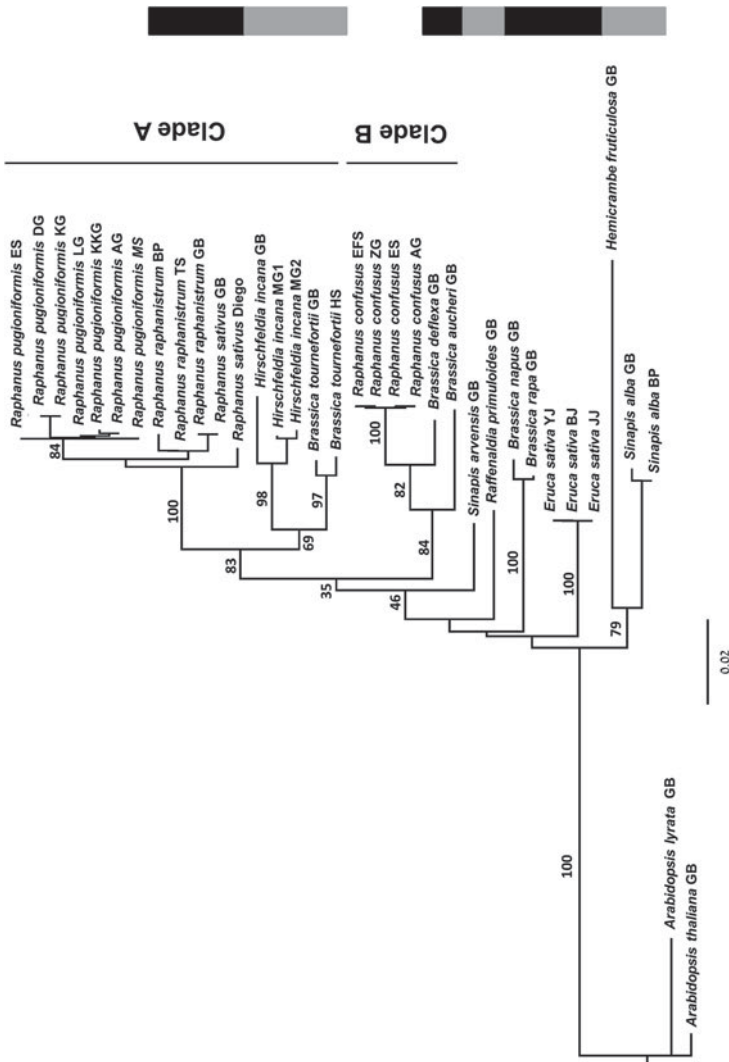


FIG. 3. PhyML maximum-likelihood tree of the relationships among *Raphanus* species. The tree is based on sequences of the nuclear ribosomal 5.8S gene and ITS region (ITS1 and partial ITS2) of the *Raphanus* species used in this study. Sequences of other members of the Brassicaceae were taken from GenBank (see Table 1). The bootstrap support values from 100 replicates are indicated above the branches. *Arabidopsis thaliana* and *A. lyrata* served as the outgroup. Clade A refers to *Raphanus* sect. *Raphanus* plus *Hirschfeldia incana* and *Brassica tournefortii*, and clade B to *Raphanus* sect. *Hesperidopsis* plus *Brassica aucheri* and *B. deflexa*. The panel on the right assigns the taxa studied to tribe Brassicaceae lineages based on cpDNA analysis [adapted from Warwick & Black (1997) and Arias & Pires (2012)]. Black: *Oleracea* lineage; grey: *Nigra* lineage; no other *Raphanus* species were treated by these studies.

and *Hemicrambe fruticulosa* are assigned to the monophyletic *Nigra* clade (Warwick & Hall, 2009; Arias & Pires, 2012). Conversely, both Warwick & Sauder (2005) and this present study, which are ITS-based, demonstrate that the *Nigra* and *Oleracea* lineages are non-monophyletic. Especially noteworthy is the uncertain relationship between *Brassica tournefortii*, *Hirschfeldia incana* and *Raphanus raphanistrum*, which appear to be closely related in the ITS analyses (Fig. 3; Warwick & Sauder, 2005), but not so in cpDNA studies (Warwick & Hall, 2009; Arias & Pires, 2012). Furthermore, the relationship between *Raphanus raphanistrum* and *Eruca* spp., which is close in the cpDNA but distant in ITS, is still to be elucidated.

In the past two decades traditional taxonomic classification of the Brassicaceae has been challenged by molecular phylogenies, suggesting that many fruit characters are homoplastic (e.g. Mummenhoff *et al.*, 2005; Hall *et al.*, 2011). Furthermore, several genera which were circumscribed by a common fruit structure have been subjected to taxonomic rearrangement after molecular phylogenies had indicated a polyphyletic origin, for example *Sisymbrium* (Warwick *et al.*, 2006a) and *Dentaria* (Sweeney & Price, 2000) [for a review see Al-Shehbaz (2012)].

Based on our preliminary molecular analysis, we suggest that the circumscription of *Raphanus* sensu Schulz (1919) as well as Warwick *et al.* (2006b) be revised. Both our ITS and the morphological data confirm that *Raphanus raphanistrum*, *R. pugioniformis* and *R. sativus* belong to a well-supported lineage circumscribed as *Raphanus*, following the generitype *R. sativus* (Jarvis, 2007). The monotypic section *Hesperidopsis*, however, does not seem to belong to the same lineage as the remaining *Raphanus* species, and we recommend transferring it to a different genus, namely *Quidproquo*, reinstating the name *Quidproquo confusum*. Thus, section *Raphanis* becomes a redundant name, as it remains the only one in the genus.

Finally, our analysis supports Baillargeon's point of view (Baillargeon, 1985a,b), treating the reduction of the valvar fruit segment as a result of parallel evolution in the genera *Raphanus* and *Quidproquo*. Molecular analyses based upon a broad taxon sampling and a multi-gene approach are needed to assign and evaluate phylogenetic relationships of *Raphanus* species and related taxa of tribe Brassicaceae.

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