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Using complementary techniques to distinguish cryptic species: A new *Erysimum* (Brassicaceae) species from North Africa[1]

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- *Premise of the study*: Cryptic species are superficially morphologically indistinguishable and therefore erroneously classified under one single name. The identification and delimitation of these species is usually a difficult task. The main aim of this study is to provide an inclusive methodology that combines standard and new tools to allow accurate identification of cryptic species. We used *Erysimum nervosum* s.l. as a model system.
- *Methods*: Four populations belonging to *E. nervosum* s.l. were sampled at their two distribution ranges in Morocco (the Atlas Mountains and the Rif Mountains). Fifteen individuals per population were collected to assess standard taxonomic traits. Additionally, corolla color and shape were quantified in 30 individuals per population using spectrophotometry and geometric morphometrics, respectively. Finally, we collected tissue samples from each population per species to study the phylogenetic relationships among them.
- *Key results*: Using the standard taxonomic traits, we could not distinguish the four populations. Nonetheless, there were differences in corolla color and shape between plants from the two mountain ranges. The population differentiation based on quantitative morphological differences were confirmed and supported by the phylogenetic relationships obtained for these populations and the rest of the Moroccan *Erysimum* species.
- *Conclusions*: The joint use of the results obtained from standard taxonomic traits, quantitative analyses of plant phenotype, and molecular data suggests the occurrence of two species within *E. nervosum* s.l. in Morocco, one located in the Atlas Mountains (*E. nervosum* s.s.) and the other in the Rif Mountains (*E. riphaeanum* sp. nov.). Consequently, we suggest that combining quantitative and molecular approaches with standard taxonomy greatly benefits the identification of cryptic species.

Key words: Atlas Mountains; Brassicaceae; corolla color; corolla shape; cryptic species; *Erysimum nervosum*; *Erysimum riphaeanum* sp. nov.; geometric morphometrics; Rif Mountains; taxonomy.

Plant taxonomy has traditionally relied on morphological trait analysis (Sivarajan, 1991). This analysis, based on the use of diagnostic traits, has been complemented in the last decades with phenetic analysis tools (Rohlf and Marcus, 1993). These morphological approaches have been very useful to describe new species, to construct keys, and to differentiate species in the field. Nevertheless, in some plant groups with low morphological differences between taxa, distinguishing species using only these morphological traits is a difficult task. Since the seminal work of Grant (1981), these assemblages of species, called species complexes, have been widely acknowledged to represent a very intriguing evolutionary problem because they probably represent lineages where speciation is recent or yet incomplete (Nosil et al., 2009; Schluter and Conte, 2009). In such situations, ascribing a newly described population to a new species will depend on the species concept used by the plant taxonomists. Under the evolutionary and phylogenetic concepts of species (Wiley, 1981; Cracraft, 1989; de Queiroz and Donoghue, 1990), this new population should be an independent monophyletic lineage to be considered as new species. In this context, DNA sequencing analysis could be crucial to diagnose the polyphyletic status in a species complex, and to recognize individual species.

Molecular techniques have helped to solve taxonomic problems when species are difficult to separate morphologically (Knowles and Bryan, 2007; Judd et al., 2008). However, those analyses can be time- and resource-consuming, making them unfeasible in many regions with poor resources where paradoxically there is much unclassified biodiversity (Hillis, 1987). In this context, the development of quantitative techniques for assessing important taxonomic traits may be very useful. Because it is very difficult to measure the whole plant phenotype, these techniques should focus on characters known to be of ecological and evolutionary significance (Roy and Foote, 1997). In this sense, traits such as corolla shape and color, widely used to

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discriminate or arrange taxa (e.g., Heywood et al., 2007), are particularly relevant. Consequently, a rising number of ecological studies have used either geometric morphometric analysis of corolla shape (e.g., Gerie et al., 1997; Medel et al., 2003; Gómez et al., 2008b, 2009) or spectrophotometric quantification of corolla color (e.g., Gaisterer et al., 1999; Whitney et al., 2009). The combination of these two not commonly used techniques may be useful to distinguish cryptic species.

Accurately detecting cryptic species may be also important for developing adequate conservation agendas. Schönrogge et al. (2002) showed the dual problem of cryptic species complexes for conservation programs: (1) the species considered for conservation would comprise more than one species, each of them more threatened than the group as a whole; (2) thus, these different species, comprising a cryptic complex, would require a more specific conservation strategy. For this reason, any technique helpful to detect cryptic species may be useful for improving our conservation strategies.

Erysimum L. (Brassicaceae) is composed of over 200 species mainly distributed in the northern hemisphere (Polatschek, 1986), having in the western Mediterranean region an important diversification center (Greuter et al., 1986). According to Koch and Al-Shehbaz (2008), this genus is centered primarily in Eurasia, with eight species inhabiting northern Africa and Macaronesia and 15 more species in North America. The genus has been traditionally placed in the broadly circumscribed Camelineae De Candolle (sensu Al-Shehbaz et al., 2006). However, recent molecular studies have suggested that *Erysimum* could be a sister genus of the tribe Descurainieae Al-Shehbaz, Beilstein & E. A. Kellogg (Beilstein et al., 2008) or could even be a unigeneric tribe, Erysimeae Dumortier (Bailey et al., 2006; Koch and Al-Shehbaz, 2008). Taxonomic problems also arise intragenerically (e.g., Faverger, 1978; Nieto Feliner, 1991), as manifested by the recognized number of *Erysimum* species, which varies between 180 to 223 species, depending on the author (Al-Shehbaz et al., 2006; Warwick et al., 2006; Koch and Al-Shehbaz, 2008). These taxonomic difficulties arise as a consequence of the morphological similarities among most *Erysimum* species, probably reflecting rapid speciation processes within the genus. These rapid speciation events generate sibling or cryptic species that, although being almost identical morphologically, are ecologically and/or geographically isolated from each other.

The main goal of this study is to test whether the joint use of standard taxonomic tools, quantitative techniques, and phylogenetic tools can improve our ability to identify cryptic species. We used standard taxonomic analysis of diagnostic traits with a geometric morphometric analysis of corolla shape, spectrophotometric determination of corolla color, and phylogenetic analysis of molecular data to identify species within *Erysimum nervosum* s.l.

MATERIALS AND METHODS

Study system—Two main mountain ranges occur in North Africa, the Atlas and the Rif (Fig. 1). The Atlas extends ca. 2400 km through Morocco, Algeria, and Tunisia. In Morocco, the Atlas is subdivided into three ranges (from north to south): Middle Atlas, High Atlas, and Anti-Atlas. In the northern Morocco, and parallel to the Mediterranean coast, the Rif Mountains cover ca. 50 000 km^2. This latter mountain range, having a geological origin common to southern Spain Baetic ranges, with which it forms the Baetic-Rifean Arc, is geologically distinct from the Atlas range (Lonergan and White, 1997).

According to the floristic studies carried out in the northern Africa (Ball, 1877; Jahandiez and Maire, 1932; Maire, 1967; Valdés et al., 2002), four autochthonous *Erysimum* taxa inhabit the area: (1) *E. incanum* Kunze, widely distributed in the region; (2) *E. semperflorens* Schousb., found in the west coast of Morocco and in the north coast, between Morocco and Algeria; (3) *E. wilczekianum* Braun.-Blanq. & Maire, inhabiting the Middle Atlas; and (4) *E. nervosum* Pomel, which inhabits the two Moroccan mountain ranges, the Atlas and the Rif Mountains. Within this latter taxon, some authors have recognized several varieties, subspecies and even species (Ball, 1877; Maire, 1967; Favarger and Galland, 1982). However, in recently published reviews, all the infraspecific categories haven been included in the *E. nervosum* species complex (Valdés et al., 2002; Koch and Al-Shehbaz, 2008).

Erysimum nervosum s.l. is a monocarpic, perennial herb endemic to the Atlas Mountains, where the species was firstly described (Pomel, 1875), and the Rif Mountains (Valdés et al., 2002). In the Atlas Mountains, it grows on oligotrophic soils (schists) in alpine and subalpine grasslands and scrublands from 1500 to 2500 m a.s.l. In contrast, the species in the Rif Mountains inhabits forest and shrubland canopies between 1200 and 1800 m a.s.l., always on basic soils (limestones). In both regions, this species is biennial, growing 2 years as a vegetative rosette and then dying after producing stalks with between a few and hundreds of yellow bisexual flowers. The flowers are self-compatible and are pollinated by a diverse assemblage of pollinators (M. Abdelaziz et al., University of Granada, unpublished data).

Between 2006 and 2009, we studied *E. nervosum* s.l. in both of the ranges where this species occurs in Morocco (i.e., Atlas and Rif Mountains) (Fig. 1). In each range, we selected two populations, which are hereafter referred to as "Ene" for the Atlas populations and "Eri" for the Rif populations (Fig. 1 and Table 1).

Standard taxonomic study—For the taxonomic study, we collected 15 plants per population, totaling 60 samples. Plants were dried, pressed, mounted on herbarium sheets, and registered at the herbarium of the University of Granada (GDA). Afterward, we measured 30 quantitative and qualitative variables that have been widely used in several floras to differentiate species in this genus (see Appendix 1). The traits were measured with digital calipers with ± 0.1 mm resolution, except plant height, which was measured with measuring tape with ± 0.5 cm resolution. These traits were compared by nested ANOVAs, including range (Atlas vs. Rif) as the main factor and population as a random factor nested within range. All statistical analyses were performed with the software JMP, version 7.0 (SAS Institute, Cary, North Carolina, USA).

Geometric morphometric analysis of corolla shape—Corolla shape was quantified in 30 randomly selected plants per population by means of landmark-based geometric morphometric tools (Bookstein, 1991; Rohlf, 2003; Zelditch et al., 2004). We took a digital photograph of one flower per plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects (Gómez et al., 2006) and always in the same position to ensure the conservation of petal homology across flowers. We defined 32 co-planar landmarks (Fig. 2) (Appendix 2), located along the outline of the flowers and the aperture of the corolla tube; the landmarks were chosen to provide comprehensive coverage of the flower shape (Roth, 1993; Zelditch et al., 2004). Landmarks were defined by reference to the midrib (landmarks 1, 9, 17, and 25), primary veins (landmarks 2, 8, 10, 16, 18, 24, 26, and 32), and secondary veins (landmarks 3, 4, 6, 7, 11, 12, 14, 15, 19, 20, 22, 23, 27, 28, 30, and 31) of each petal as well as the connection between petals (landmarks 5, 13, 21, and 29) (Fig. 2). We captured the landmarks using the software tpsDig version 1.4 (available at the Stony Brook Morphometrics website: http://life.bio.sunysb.edu/morph/morphmet.html). Afterward, the two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the generalized Procrustes analysis (GPA) superimposition method (Rohlf and Slice, 1990; Slice, 2001).

Differences in corolla shape between Atlas and Rif populations were quantified by means of a canonical variate analysis (CVA) (Zelditch et al., 2004; Klingenberg and Monteiro, 2005). The CVA is a specific multivariate analysis that optimizes between-group differences relative to within-group variation. It generates several CV axes and computes the Procrustes distances among groups in the CV space. We additionally performed a Procrustes discriminant analysis, which examines the separation between two groups of observations. These two types of analyses are complementary because discriminant analysis is more useful for comparisons of specific groups, whereas CVA may be more useful for general analysis of group structure in a data set. The statistical significance

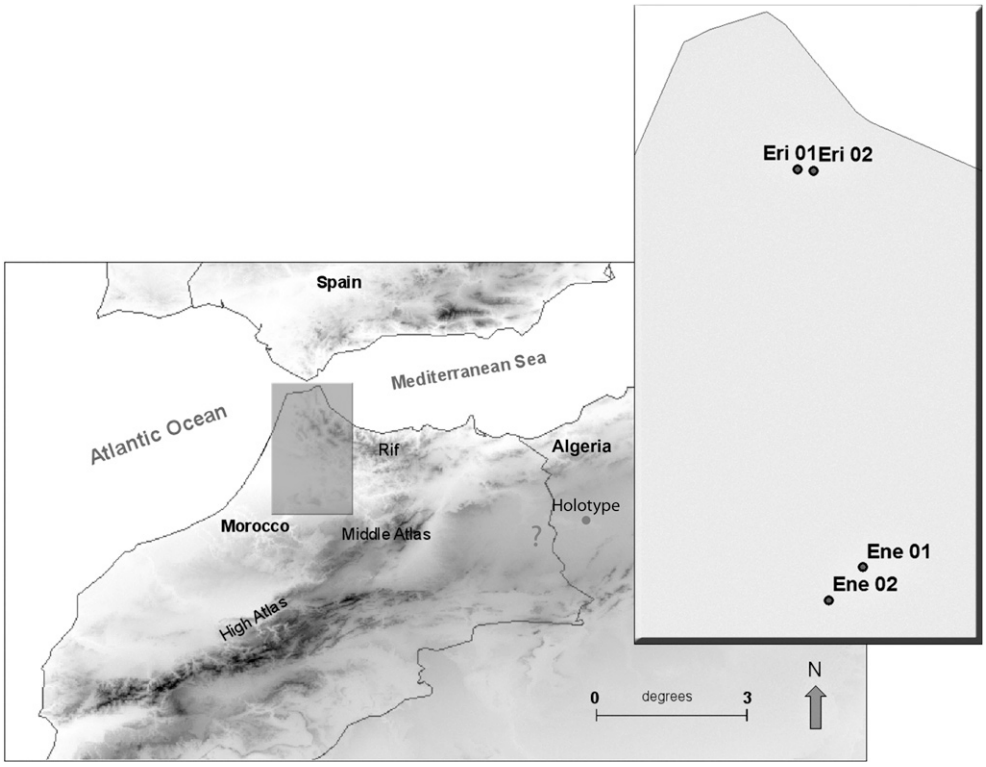
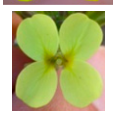


Fig. 1. Location of the studied populations of the *Erysimum nervosum* complex in Morocco. ? = Locations unsuccessfully prospected; Holotype = Location of the holotype (Pomel, 1875).

of the between-groups Procustes distances was determined by randomization tests using 10 000 permutations with the software MorphoJ (Klingenberg, 2008).

Corolla color analysis—The corolla color was quantitatively measured in situ in each plant used in the geometric morphometric study by means of spectrophotometry, using an USB4000 miniature fiber optic spectrometer with a USB-DT deuterium tungsten halogen source (Ocean Optics, Dunedin, Florida, USA). This method has several advantages over the traditional visual evaluation. Namely, it gives accurate and objective measurements of reflectance (i.e., spectral reflectance curve) over the entire color spectrum including ultraviolet (300–700 nm), and the data can be stored automatically in computer spreadsheets (Chittka and Kevan, 2005). Following Vorobyev and Osorio (1998) and Montgomerie (2006), we used a hue–saturation–brightness (HSB) color assessment model (Andersson and Prager, 2006; Sharma, 2004) to characterize the corolla color of the studied populations by calculating brightness, chroma, and hue. Brightness, an achromatic measure that shows the maximum reflectance, was measured as the cumulative reflectance values of the entire spectrum (Andersson and Prager, 2006; Montgomerie, 2006). Chroma, which is an estimate of a color purity and perceived intensity, was calculated as the difference between the maximum and minimum reflectance values divided by the average reflectance (Andersson and Prager, 2006; Montgomerie, 2006). Hue is the

TABLE 1. Location, habitat type, and flower appearance of the studied populations of the *Erysimum nervosum* complex. Flower photos were chosen to show the average shape in each population.

Population	Mountain range	Latitude	Longitude	Altitude (m a.s.l.)	Habitat type	Flower appearance
Ene 01	Atlas	33°26.308′	−4°56.188′	1711	Perennial grassland	
Ene 02	Atlas	33°17.661′	−5°5.159′	1802	Perennial grassland	
Eri 01	Rif	35°11.14′	−5°13.32′	1650	Open forest	
Eri 02	Rif	35°10.742′	−5°9.106′	1398	Shrubland	

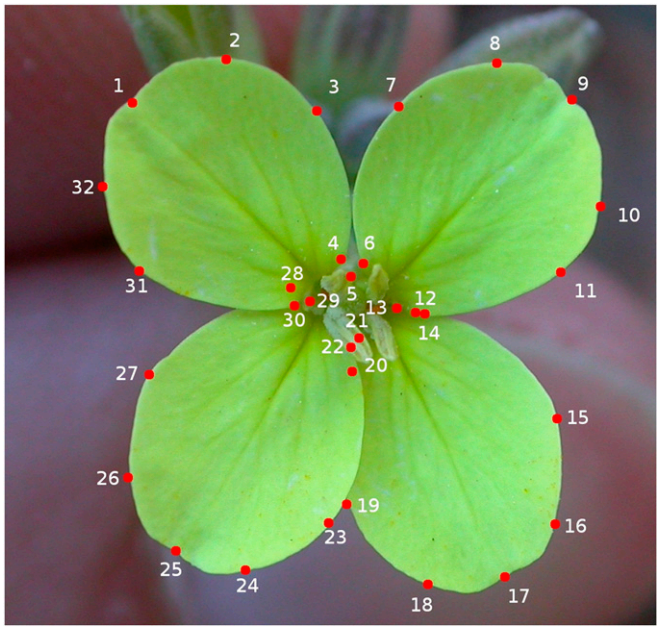


Fig. 2. A planar view of the *Erysimum nervosum* s.l. corolla, showing the location of the 32 landmarks used in the geometric morphometric analysis.

degree to which a stimulus can be described as similar to, or different from, stimuli that are described as red, green, blue, or yellow. Hue was estimated as the wavelength with maximum reflectance (Andersson and Prager, 2006; Montgomerie, 2006). Between-population differences in color parameters were quantified by one-way ANOVAs with Tukey–Kramer honestly significant difference (HSD) post hoc comparison.

Analysis of phylogenetic relationships—We collected fresh leaf tissue material from each population (Table 1). In addition, we also collected fresh tissue from the other Moroccan *Erysimum* species (*E. incanum*, *E. semperflorens*, and *E. wilczekianum*). This material was dried and conserved in silica gel until DNA extraction. We extracted DNA by using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA) with at least 60 mg of plant material crushed in liquid nitrogen.

We amplified four different DNA regions: two plastid (*ndhF*, ~2000 bp and *trnT-L*, ~1300 bp) and two nuclear (ITS1, ~350 bp and ITS2, ~350 bp). We used the primers ndhF5 and ndhF2100 (Olmstead and Sweere, 1994) to amplify *ndhF*; tabA and tabD (Taberlet et al., 1991) for *trnT-L*; ITS1 and ITS2 primers for the ITS1 region (White et al., 1990); ITS3 and ITS4 primers for the ITS2 region (White et al., 1990). PCR reactions were performed in a total volume of 50 µL, with the following composition: 5 µL 10× buffer containing $MgCl_2$ at 1.5 mmol/L (New England BioLabs), 0.1 mmol/L each dNTP, 0.2 µmol/L each primer and 0.02 U *Taq* DNA polymerase (New England Biolabs). PCRs were performed in a Gradient Master Cycler Pro S (Eppendorf, Ibérica, Spain) using a initial denaturing step of 3 min at 94°C and a final extension step of 3 min at 72°C in all the reactions. Reactions for *ndhF* included 35 cycles of 94°C for 15 s, 47°C for 30 s, and 72°C for 90 s. Reactions for *trnT-3′trnL* included 35 cycles (94°C 15 s, 53°C 30 s, and 72°C 90 s). Reactions for ITS1 also included 35 cycles (94°C 15 s, 64°C 30 s, and 72°C 45 s). For ITS2, reactions included 35 cycles of 94°C 15 s, 53°C 30 s, and 72°C 45 s).

PCR products were mixed with 0.15 volume of 3 mol/L sodium acetate, pH 4.6 and 3 volumes 95% (v/v) ethanol and subsequently purified by centrifuging at 4°C. Amplicons were then sent to Macrogen (Maryland Rockville, USA) to be sequenced using the respective PCR primers and additional internal primers for *ndhF* (ndhF-599, ndhF-989-R, and ndhF-1354) and *trnT-L* regions (tabB and tabC).

Chromatograms were reviewed using the program Finch TV v1.4.0 (Geospiza, Seattle, Washington, USA) and the sequences edited using the program BioEdit v7.0.5.3 (Hall, 1999; Larkin et al., 2007). For the outgroup, we used *Arabidopsis thaliana* sequences from GenBank. This species was used because it is a close relative of *Erysimum* (Al-Shehbaz et al., 2006). We tested for incongruence between the nuclear and plastid genes using an incongruence length difference (ILD) test (Farris et al., 1995) as implemented in the program ILD-bionj v1.0 (Zelwer and Daubin, 2004); phylogenetic data for the two sequence types were not significantly incongruent ($P = 0.528$). Sequences of different markers were concatenated on an individual basis and then aligned using the ClustalW (Thompson et al., 1994) tool in BioEdit (Hall, 1999; Larkin et al., 2007). The sequences reported in the present study have been deposited in GenBank (Appendix 3).

Alignments were manually reviewed, and a region of indels and a string of adenines in the *trnT-L* (positions 2880–3300 of the concatenated alignment) were deleted using the GBlocks Server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana, 2000) with the less stringent selection.

We built phylogenetic trees using both maximum likelihood (Felsenstein, 1973) with the program PhyML v2.4.4 (Guindon and Gascuel, 2003) and Bayesian Markov chain Monte Carlo (MCMC) inference (Yang and Rannala, 1997) using the program MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The PhyML analysis was performed with default options and assuming a general time reversible (GTR) model. This was the best fitting evolutionary model for the four concatenated regions as estimated by the program ModelTest v3.7 using the Akaike information criterion (AIC) (Akaike, 1974; Posada and Crandall, 1998). Base frequencies, the proportion of invariable sites, substitution rates, and the alpha parameter of the gamma distribution were estimated by PhyML. Branch support was calculated both with the approximate likelihood ratio test (SH-like supports option) and the bootstrap (Felsenstein, 1985; 1000 replicates). For Bayesian analysis, we used MrBayes in the online Bioportal of the University of Oslo (http://www.bioportal.uio.no/), partitioning the data into four regions, one for each locus cited (ITS regions treated as a single locus), and we estimated the best fitting evolutionary model for each region using MrModelTest v2.3 (Nylander, 2004). Analysis lasted for 4 million MCMC generations, with a sample frequency of every 100 generations, and we removed the first 25% of trees as burn-in, after checking trace files with the program Tracer v1.4 (Rambaut and Drummond, 2007) to determine the convergence of the two independent Bayesian MCMC runs. The consensus trees were visualized, edited, and exported using the program MEGA v4.0.2 (Tamura et al., 2007).

RESULTS

Standard taxonomic study—According to the nested ANOVAs, only one quantitative trait (petal width) significantly differed between the Atlas and Rif populations (Table 2 and Appendix 4). Similarly, no differences were found for the qualitative traits, with the exception of a marked dark rib in the fruit, which is less conspicuous in Rif populations (Table 3).

Geometric morphometric analysis of corolla shape—The two main canonical variate axes accounted for 90% of the variance

TABLE 2. Results of the comparison between *Erysimum nervosum* s.l. populations from Rif and Atlas region (mean ± SE) for quantitative morphological traits. *F*-ratios refer to nested ANOVA, using population as a random effect (results not shown), df = 3; ns = not significant ($P > 0.05$).

Trait	Atlas ($N = 30$)	Rif ($N = 30$)	*F*-ratio	*P*
Number of stems	9.23 ± 1.03	4.81 ± 0.97	7.56	ns
Plant height (cm)	20.20 ± 0.74	18.21 ± 1.10	0.27	ns
Leaf length (mm)	14.54 ± 1.24	21.82 ± 1.45	9.48	ns
Leaf width (mm)	0.85 ± 0.047	1.22 ± 0.09	6.53	ns
Number of flower	43.92 ± 5.65	34.44 ± 6.53	0.47	ns
Sepal length (mm)	7.69 ± 0.14	8.53 ± 0.25	1.46	ns
Petal length (mm)	13.87 ± 0.31	15.04 ± 0.31	4.86	ns
Petal width (mm)	2.96 ± 0.11	3.72 ± 0.17	63.89	<0.0001
Filament length (mm)	8.99 ± 0.17	9.39 ± 0.15	2.71	ns
Number of fruits	23.92 ± 3.65	13.19 ± 2.72	2.66	ns
Length of fruit pedicel (mm)	2.79 ± 0.10	3.53 ± 0.19	12.58	ns
Fruit length (mm)	14.50 ± 0.97	18.05 ± 1.79	0.62	ns
Fruit width (mm)	0.55 ± 0.30	0.65 ± 0.04	0.61	ns

TABLE 3. Results of the comparison between *Erysimum nervosum* s.l. populations from Rif and Atlas Mountains (mean ± SE) for qualitative morphological traits.

Trait	Atlas ($N = 30$)	Rif ($N = 30$)
Life cycle	Monocarpic perennial	Monocarpic perennial
Stem shapes	Erect to ascending	Erect to ascending
Plant surface	Hairy	Hairy
Hair shape	All medifixed	All medifixed
Lower leaves arrangement	Rosette-forming	Rosette-forming
Lower leaves	Simple and entire	Simple and entire
Cauline leaves	Simple and entire	Simple and entire
Base of cauline leaves	Sessile	Sessile
Inflorescence type	Simple	Simple
Inflorescence position	Terminal	Terminal
Stigma shape	Bilobed	Bilobed
Indument of fruit pedicel	Hairy	Hairy
Fruit rib	Dark-marked	Slightly marked
Fruit patent	Erect	Erect
Persistence of fruits	Deciduous	Deciduous
Valve surface	Hairy	Hairy

in corolla shape (Fig. 3). As Fig. 3 shows, the two Atlas populations did not differ according to their Procrustes distance in the canonical variate space, but they did differ from the Rif populations. The two Rif populations, however, differed in the canonical variate space, although the variation was mainly through the second axis. Discriminant analyses outcomes were similar to that of canonical variate analyses and are not shown.

Corolla color analysis—We obtained similar spectral profiles for populations belonging to the same mountain range, but considerable differences between mountain ranges. The spectral profile of the flowers was very different between Rif and Atlas populations (Fig. 4). Moreover, a reflectance peak obtained at ca. 450–475 nm for the Rif populations was completely absent for the Atlas populations. Brightness and chroma also statistically differed among the four studied populations, although they were more similar between populations belonging to the same range (Fig. 4). In contrast, hue was only significantly different for one of the populations (Fig. 4).

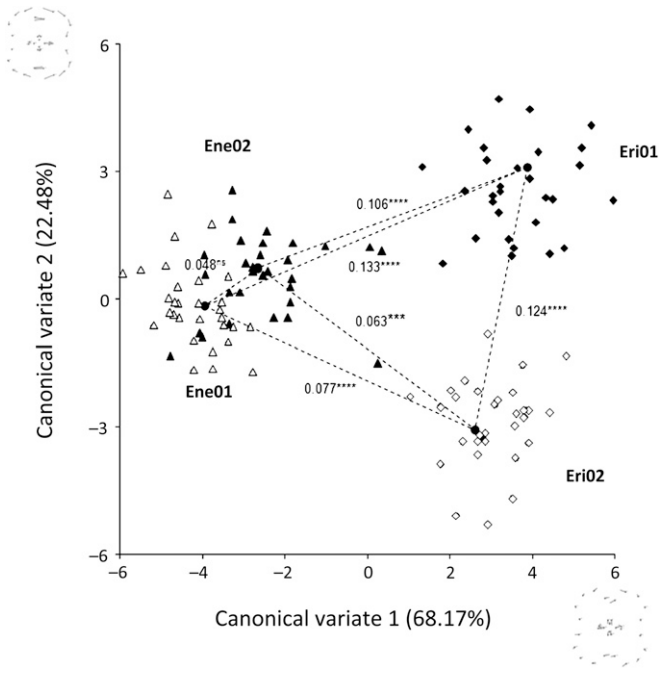


Fig. 3. Results of the canonical variate (CV) analysis. The figure represents the position of the plants belonging to each of the four studied populations in the two-axis CV space. The variance in shape explained by each CV axis, the change in shape produced by each axis, and the Procrustes distances between each population (ns, nonsignificant; ***, $P < 0.001$; ****, $P < 0.0001$) are shown.

Phylogenetic analysis—The topologies of the phylogenetic trees using maximum likelihood and Bayesian inference approaches were similar (Fig. 5). The only difference between them is the position of one *E. incanum* population (Ei03, Fig. 5). The Rif populations of *E. nervosum* (Eri01 and Eri02) were clearly separated from the Atlas populations of *E. nervosum* (Ene01 and Ene02). In fact, the Atlas populations were always associated with *E. semperflorens* forming a clade, and they never appeared to be sister to Rif populations. These relationships are strongly supported by bootstrap, approximate likelihood ratio test values, and posterior probabilities (Fig. 5). Therefore, Rif and Atlas populations appear to represent two different evolutionary lineages.

DISCUSSION

Our study identifies three main points that may be useful when trying to identify cryptic species. First, it seems that standard taxonomic traits could be uninformative in some study systems. Here this standard phenotypic analysis revealed weak differences in the morphology of *E. nervosum* plants in the two distribution areas because they differed in only one quantitative trait and one qualitative trait. This outcome reflects the difficulty of discriminating between Atlas and Rif populations and opens the possibility that they are potential cryptic species.

Second, we have seen that using quantitative complex traits, which may be important during the evolutionary divergence process of species pairs, could be also useful to distinguish some groups of populations. Under these circumstances, additional approaches can allow a better identification and determination of species within syngameons and cryptic species complexes (Bickford et al., 2007). In this sense, corolla shape and color could be used as important taxonomic key characters, not only when the differences are evident (e.g., zygomorphic flower vs. actinomorphic flower, red corolla vs. white corolla), but also when they are subtle and quantitative. Most *Erysimum* species have a yellow corolla with similar shapes which, in principle, makes these traits somewhat subjective and difficult to use for differentiating closely related species. However, the approach used in the present study, in which both corolla shape and color were quantitatively measured by geometric morphometrics and field spectrophotometry, respectively, seems to be useful for discerning groups in those cases where the standard taxonomic tools are insufficient. These two traits, furthermore, are especially useful in *Erysimum* because they are associated with pollination and reproductive success in many species and they do not change whether measured in the field or in the greenhouse.

The use of corolla shape and color has further interest since they are known to have important evolutionary and ecological implications in many plant species (Dyer, 2004; Chittka et al., 1999; Schemske and Bradshaw, 1999). We have previously

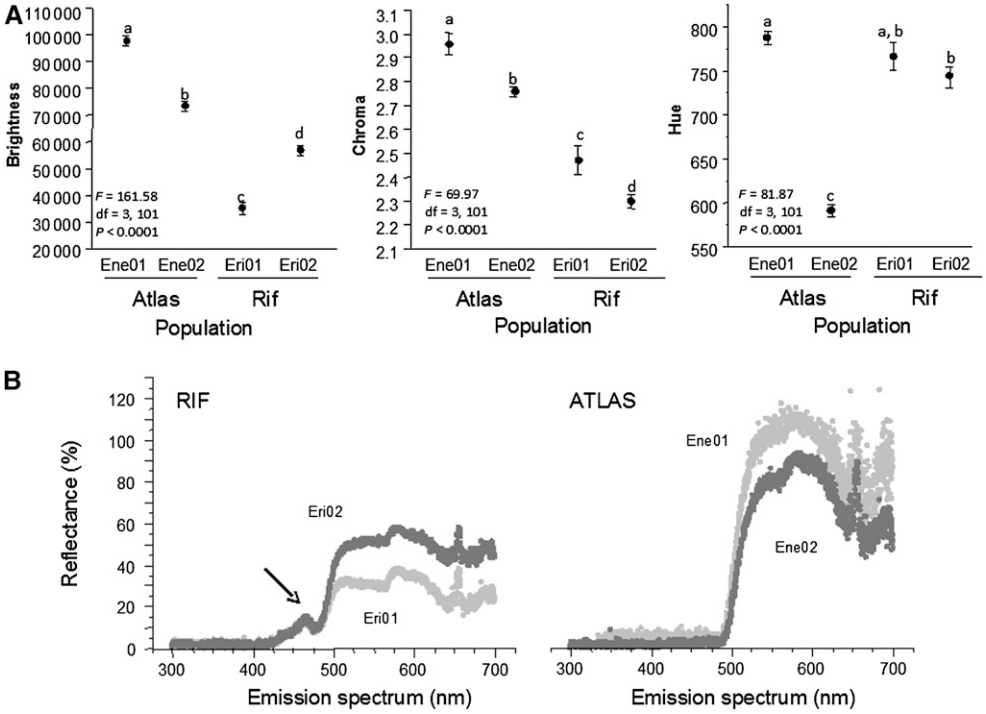


Fig. 4. (A) Mean values for brightness, chroma, and hue per each population in the two studied regions. *F*-ratios refer to one-way ANOVAs. Letters indicate the groups where the differences are significant, according to a Tukey HSD comparison. (B) Comparative spectral profile for the percentage of reflectance for each population in the two studied regions. The arrow shows a local maximum obtained in the populations from the Rif Mountains and not present in the populations from Atlas Mountains.

shown that corolla shape is under pollinator-mediated selection, playing an important role in the adaptation to their pollinators of some *Erysimum* species from the Iberian Peninsula (Gómez et al., 2006, 2008a, b, 2009). Because the selective pressures exerted by pollinators seem to be similar across many *Erysimum* species (Gómez et al., 2006, 2008a, b, 2009; Gómez and Perfectti, 2010; Ortigosa and Gómez, 2010), these complex traits will presumably also be similar among different species. Consequently, the study of these traits may help to disentangle the evolutionary divergence resulting in morphological differences between closely related species.

The analysis of quantitative traits in common garden or greenhouse conditions may be crucial in some occasions, since it allows distinguishing between genetically controlled and environmentally controlled variance. However, the traits quantified in the present study, corolla shape and color, are identical between greenhouse and natural populations of numerous *Erysimum* species that we are presently growing, including one species

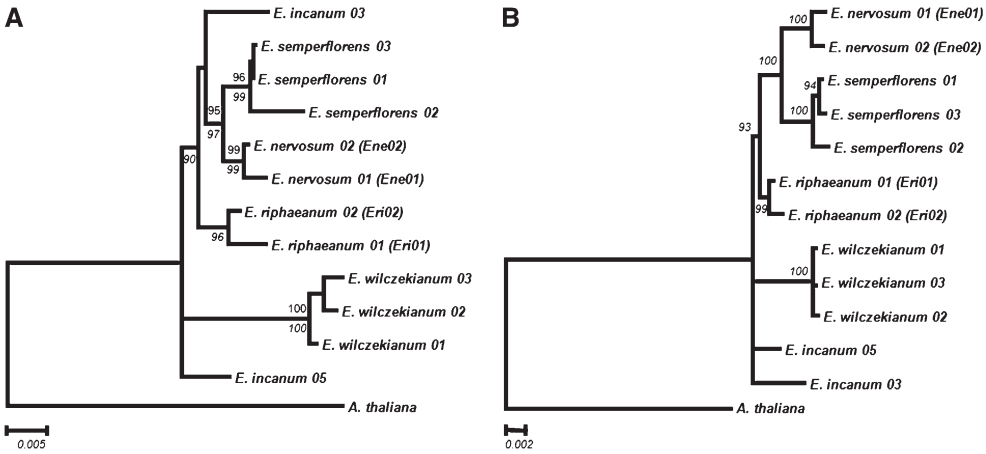


Fig. 5. Phylogenetic position of *Erysimum nervosum* and *E. riphaenum* populations within the North African species of the genus *Erysimum*. (A) Maximum likelihood tree obtained with PhyML; branch reliability supports, calculated by approximate likelihood ratio test, appear below branch lines, and bootstrap values above branch lines. (B) Tree obtained using Bayesian Markov chain Monte Carlo inference; branch supports values are posterior probabilities. Only branch supports higher than 75% are shown.

analyzed in this study, *E. semperflorens* (authors' personal observation). For this reason, we think *E. nervosum* and *E. riphaeanum* behave as the other congenerics and will display similar corolla shape and color in field and greenhouse conditions.

Finally, we have shown that molecular data may be used to corroborate what has been found with previous data. Molecular techniques (mainly molecular phylogenies) are very useful for identifying and describing new species, but they are not a panacea for delimiting species in some complex situations involving cryptic species (Bickford et al., 2007). However, such techniques have proven very useful for identifying cryptic species with polyphyletic origins, as shown in our present study. This kind of nonsister cryptic species can be produced by phenotypic convergence, in which the different species have similarly responded to the same selective pressures (Futuyma, 1997; Keller and Lloyd, 1992), or by phenotypic stasis, which is common for traits undergoing selective pressures from generalist interactions (Williamson, 1987). However, molecular phylogenetic analyses based on only a few sequences could fail, identifying recently derived sibling species, since these species usually show insufficient sequence variation (Rubinoff et al., 2006) or incomplete lineage sorting (Maddison and Knowles, 2006).

There are several advantages of using complementary techniques to identify cryptic species in plants. First, they could unravel hidden biodiversity not previously detected (Blaxter, 2004). We presume the existence of many undescribed and undetected cryptic species in plants, since cryptic plant species have received less attention than cryptic animals species (e.g., Schönrogge et al., 2002; Bickford et al., 2007; Pfenninger and Schwenk, 2007). Second, the use of complementary techniques can help to identify the mechanisms responsible for the formation of cryptic species. Combining the study of phylogenetic relationships with the analysis of the adaptive role of traits contributing to the speciation may be useful to discern among evolutionary convergence, phenotypic stasis, or cryptic speciation. In our case, since *E. riphaeanum* and *E. nervosum* are not sister species in the molecular phylogeny, they were not produced by cryptic speciation. Third, from an ecological point of view, the identification of species in cryptic complexes is fundamental to accurately establish the degree of generalization/specialization in the ecological interactions of those species (Molbo et al., 2003). A nominal species previously categorized as a generalist could actually be a group of specialist cryptic species. In our case, the pollination system of the two *Erysimum* cryptic species appears much more generalist if we erroneously consider them as one species (M. Abdelaziz et al., unpublished data).

The dual problem of cryptic species complexes for conservation programs showed by Schönrogge et al. (2002) suggests that an accurate determination of the taxonomy of a given group is a first step for the establishment of efficient conservation policies (Leadlay and Jury, 2006; Bickford et al., 2007). Our results illustrate this matter, showing that the widely distributed species *E. nervosum* s.l. is actually two species, one of which, *E. riphaeanum*, is narrowly distributed and therefore more prone to extinction (Bickford et al., 2007). Considering the scarce knowledge about the population features of the new species, *E. riphaeanum* must be considered data deficient (DD), according to IUCN (2001). Nevertheless, due to its restricted distribution area (less than 2000 km^2) and its severely fragmented populations, the most plausible category for this species is vulnerable (Vu) (IUCN, 2003).

The Rif Mountains are one of the most important Mediterranean glacial refugia in North Africa (Battandier, 1894; Haffer, 1982), and consequently, they represent a biodiversity hotspot structured by dramatic climatic cycles (Médail and Diadema, 2009). In spite of this high biodiversity, the endangered flora of the Rif Mountains is poorly known. To date, there is only a preliminary, still uncompleted, Red List of endangered, rare and endemic plants of Morocco (Fennane and Tattou, 1998). In the last decade, some taxonomic and ecological studies have been conducted in the area (e.g., Valdés et al., 2002), and it is urgent to update and extend this list by incorporating these results as a basis to prioritize conservation measures. Hence, *E. riphaeanum* forms part of the rich biodiversity of these mountains and could benefit from conservation programs.

In addition to biodiversity conservation, ecological interactions also have important conservation interest (Kearns et al., 1998; Bronstein et al., 2004). *Erysimum riphaeanum* acts as an important node in the interaction network between plants and their pollinators because this species has a generalized and highly diverse pollinator assemblage (M. Abdelaziz et al., unpublished data). Consequently, from a conservation point of view, the species is of special interest given its direct and indirect effects on the biodiversity of these mountains.

In summary, this study shows that the combination of different morphological and molecular analyses can facilitate the identification of cryptic species, help in the design of conservation policies, and be useful for studying the evolutionary processes taking place in these recently diverged taxa.

Description of a new species—The results gathered from morphological traits, quantitative corolla color, corolla shape and phylogenetic data, led us to conclude that populations from the western Rif Mountains constitute a new species, clearly separately from *E. nervosum* Pomel. A dichotomous key of Moroccan species of *Erysimum* is included in Table 4.

Erysimum riphaeanum J. Lorite, M. Abdelaziz, A. J. Muñoz-Pajares, F. Perfectti & J. M. Gómez, **sp. nov.**

Diagnosis—Hemicriptophytum caespitosum 15–25 cm altum. Caules floriferi (2)3–5(6), erecti vel adscendentes, plerumque simplices, sparse foliosi, pilis navicularibus raris praediti. Flores in racemo c. 20–40-floro, terminali atque simplici compositi, actinomorfi, tetrameri; sepalis 8–9 mm longis, viridi-flavis; petalis 13–16 mm item longis, longe ungulatis

TABLE 4. Key to the genus *Erysimum* in Morocco.

1.	Biennial or perennial.	2
1′.	Annual.	4
2.	White or white- yellowish flowers. Stems markedly woody.	*E. semperflorens* Schousb.
2′.	Yellow flowers. Stems slightly woody at the base	3
3.	Silique with a dark-marked rib. Deep-yellow flowers. Atlas and Middle Atlas mountains.	*E. nervosum* Pomel
3′.	Silique with a not dark-marked rib. Light-yellow flowers. Rif mountains.	***E. riphaeanum* sp. nov. Lorite et al.**
4.	Leaves pinnately lobed.	*E. wilczekianum* Braun-Blanq. & Maire
4′.	Leaves dentate.	*E. incanum* Kunze

flavisque; staminibus 6, tetradynamis, filamentis 9–10 mm longis. Fructus quidem siliquosi, plus minusve nervosi, pilis navicularibus induti, erecti, 16–19 mm longi, pedicello autem 3–4 mm longo atque stigmate bilobo aut capitato. A simili specie nostra, *E. nervosum* Pomel nerviis prominentibus atque valde obscuris praecipue aperteque differt. Floret a mense Aprili usque ad mensem Iunium, fructificat autem a Iunio usque ad Iulium.

Holotype—Morocco: western Rif, Chefchaouen, jbel Talassemtane. Coord: 35°10.742′N, 5°9.106′W, 1398 m a.s.l. 17.VI.2009, Leg: M. Abdelaziz & A. J. Muñoz-Pajares, collection number: Eri010801 (GDA 55655).

Description—Hemicriptophyte caespitose of 15–25 cm height, with erect to ascendent (2)3–5(6) flowered stems, usually not ramified, sparsely leafy, with dispersed medifixed hairs. Leafs of 15–20 × 1–2 mm, linear, entire, sessile with medifixed hairs. Flowers arranged in simple and terminal racemes with ca. 20–40 flowers, actinomorphic, hermaphroditic, and tetrameric. Sepals of 8–9 mm length, green-yellowish; petals of 13–16 mm length, long clawed, light-yellow. Six tetradynamous stamens with long filaments of 9–10 mm. Fruits in siliques of 16–19 mm length, erect, pubescent, with pedicels of 3–4 mm length and stigma bilobed to capitate. This new species has a less conspicuous dark-marked rib in the fruit than *E. nervosum*.

Flowering time—April–June.

Fruiting time—June–July.

Habitat description and distribution—Inhabits gaps of holm-oak (*Quercus ilex* subsp. *ballota*) and fir (*Abies pinsapo* subsp. *maroccana*) in forests and shrublands from 1200–2800 m a.s.l. on limestone. The species is distributed along the western Rif Mountains, being more abundant in the Talasemtane massif.

Etymology—The name *E. riphaeanum* refers to the Rif Mountains (Northern Morocco), where the species is endemic.

Other observed specimens—***Erysimum nervosum;*** Algeria: O. Ghar-Rouban, 10-06-1879, A. N. Pomel, MPU005845 Holotype. Algeria: O. Aïn-Ghoraba près Terni, 06-1875, A. N. Pomel, MPU005844 Syntype?. Morocco: Medio Atlas, P. N. de Taza, paredones sobre calizas, 6-VII-1986, G. Blanca, M. Cueto, J. Garrido, C. Morales, J. F. Mota & A. Ortega, GDAC29192. MOROCCO: Ouarzazate à proximité du Tizi n' Melloul (Jab. Siroua), lat. 30.78, long. −7.6, 05-06-1980, A. Charpin, J. Fdez. Casas, F. Jacquemoud & D. Jeanmonod, MA395675-1. Morocco: High Atlas, S. From Marrakech, 2 km below ski resort of Oukaïmeden on road to Vallée de l'Ourika. 2500 m, 31.22 −7.85 1997-07-26 S. L. Jury & al., MA617106-1. Morocco: Ksar-es-Souk. Akeimeden, versant N au dessus de la MF de Midkane prés de la MF, 2100 m, lat. 32.52, long. −4.97, 09-06-1980, A. Charpin, F. Jacquemoud & D. Jeanmonod, MA395601-1. Morocco: Middle Atlas, Azrou; 1 km along road to Ain Leuh from junction with main Azrou—Midelt road above Azrou, 1650 m, 1997-07-14, S. L. Jury, A. Abaouz, M. Ait Lafkih & A.J.K. Griffiths, MA614866-1. Morocco: Medio Atlas, Carretera Ifrane a Boulemane, praderas de alta montaña sobre caliza, coord. 33°26.308′N, 4°56.188′W, 1711 m, 16-05-2009, Abdelaziz & A. J. Muñoz, GDA55658 (16 samples). Morocco: Medio Atlas, Carretera Azrou a Timahdite, coord. 33°17.661′N, 5°5.159′W, 1802 m, 16-05-2009, Abdelaziz & A. J. Muñoz, GDA55657 (14 samples).

***E. riphaeanum* sp. nov.;** Morocco: Rif, In collibus inter montes Kalaa et Tisuka, 1500 m a.s.l., 15-VI- 1928-1932 (*Iter Maroccanum*, year not specified), Font Quer, (GDA28249) (sub *E. grandiflorum*). Morocco: Chefchaouen, Djbel Talmssemtane, 1699 m, M. C. García, M. A. Mateos, F. J. Pina & I. Sánchez, 25-07-1996, SEV155811-1 (sub *E. nervosum*). Ibidem SEV155811-1 (sub *E. nervosum*). Morocco: Chefchaouen, Djbel Bouhalla, 1230 m., M. A. Mateos, A. Ortega & F. J. Pina, 25-07-1995, SEV218136-1 (sub *E. nervosum*). Morocco: Rif occidental, jbel Talassemtane, claros de matorral sobre calizas, coord. 35°10.742′N, 5°9.106′W, 1398 m, 15-05-2009, M. Abdelaziz & A. J. Muñoz-Pajares, GDA55669. Ibidem, GDA55699 (two samples). Ibidem GDA55671. Ibidem GDA55670. Morocco: Rif occidental, jbel Lakraa, claros de matorral sobre calizas, coord. 35°11.14′N, 5°13.32′W, 1650 m, 17-05-2009, M. Abdelaziz & A. J. Muñoz-Pajares, GDA55656 (10 samples).

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APPENDIX 1. Summary of the morphological traits measured in the four selected populations of the *E. nervosum* s.l. Variables cover the morphological traits observed in the genus *Erysimum* from the western Mediterranean region.

Variable	Values
1. Life cycle	1 = Annual; 2 = Biennial; 3 = Monocarpic perennial
2. Number of stems	Number of stems
3. Stem shapes	1 = Erect; 2 = Erect to ascending; 3 = Ascending; 4 = Otherwise.
4. Plant height	Maximum height in cm
5. Plant surface	1 = Glabrous; 2 = Sparsely hairy; 3 = Hairy
6. Hair shape	1 = All medifixed; 2 = Mostly medifixed; 3 = Medifixed with stellate mixed; 4 = Mostly stellate; 5 = All stellate
Leaf characters	
7. Lower leaves arrangement	1 = Rosette-forming; 2 = No rosette-forming
8. Lower leaves	1 = Simple and entire; 2 = Variously serrate; 3 = Lobed or pinnatisect
9. Cauline leaves	1 = Simple and entire; 2 = Variously serrate; 3 = Lobed or pinnatisect
10. Cauline leaves base	1 = Sessile; 2 = Petiolate; 3 = Otherwise (Amplexicaul, Decurrent, Ligulate or Perfoliate)
Inflorescence characters	
11. Inflorescence type	1 = Simple; 2 = Ramified
12. Inflorescence position	1 = Terminal; 2 = Terminal and axillary; 3 = Axillary
Flower characters	
13. Number of flowers	Number of flowers
14. Sepal length	Mean length in mm
15. Petal color	1 = White; 2 = Light yellow; 3 = Yellow, 4 = Purple
16. Petal length	Mean length in mm
17. Petal width	Mean width in mm
18. Filament length	Mean length in mm
19. Stigma shape	1 = Capitate; 2 = Capitate to bilobed; 3 = Bilobed
Fruit characters	
20. Fruit pedicel length	Mean length in mm
21. Fruit pedicel indument	1 = Glabrous; 2 = Glabrous to hairy; 3 = Hairy
22. Fruit length	Mean length in mm
23. Fruit width	Mean width in mm
24. Number of seeds in fruit	Mean number of seeds per fruit
25. Fruit patent	1 = Erect; 2 = Erect to spreading; 3 = Spreading; 4 = Adpressed to the stem
26. Fruit persistence	1 = Deciduous; 2 = Persistent
27. Valve surface	1 = Glabrous; 2 = Glabrous to slightly hairy; 3 = Hairy
Seed characters	
28. Seed length	Mean length in mm
29. Seed width	Mean width in mm

APPENDIX 2. Description of landmarks definition in genus *Erysimum*. Dividing the flower in four quadrants and following the trigonometric name for each one, here we define the landmark used for the study of corolla shape (see Fig. 2).

Landmark name	Location
1 (quadrant 2), 9 (q. 1), 17 (q. 4),25 (q. 3)	Intersection of midrib (if necessary, its continuation) and petal margin
2 (q. 2),10 (q. 1), 18 (q. 4), 26 (q. 3)	Intersection between first primary veins on the right side of the midrib (if necessary, its continuation) with the petal margin
32(q. 2), 8 (q. 1), 16 (q. 4), 24 (q. 3)	Intersection between first primary veins on the left side of the midrib (if necessary, its continuation) with the petal margin
3(q. 2), 11(q. 1), 19(q. 4), 27(q. 3)	Intersection of secondary veins on right side of the midrib (if necessary, its continuation) and the petal margin
31(q. 2), 7(q. 1), 15(q. 4), 23(q. 3)	Intersection of secondary veins on left side of the midrib (if necessary, its continuation) and the petal margin
4(q. 2), 12(q. 1), 20(q. 4), 28(q. 3)	Point where petal inflects to corolla on right side of midrib
30(q. 2), 6(q. 1), 14(q. 4), 22(q. 3)	Point where petal inflects to corolla on left side of midrib
5, 13, 21, 29	Point where both petals contact the sepals.

APPENDIX 3. Origin of the material used in the phylogenetic analyses and GenBank accession numbers.

TAXON; Population code; GenBank accessions: ITS1; ITS2; ndhF; tabAD; *Voucher specimen*, Collection locale; Herbarium.

Arabidopsis thaliana (L.) Heynh.; X52322; X52322; AP000423; AP000423.

Erysimum incanum Kunze; Ei03; HM235723; HM235735; HM235747; HM235759; *GDA56843*; Morocco, Ifrane; GDA. ***E. incanum***; Ei05; HM235724; HM235736; HM235748; HM235760; Morocco, Chefchaouen; GDA. ***E. nervosum*** Pomel; Ene01; HM235725; HM235737; HM235749; HM235761; *GDA55657*; Morocco, Ifrane; GDA. ***E. nervosum***; Ene02; HM235726; HM235738; HM235750; HM235762; *GDA55658*; Morocco, Ifrane; GDA. ***E. riphaeanum*** sp. nov.; Eri01; HM235727; HM235739; HM235751; HM235763; *GDA55655*; Morocco, Chefchaouen; GDA. ***E. riphaeanum*** sp. nov.; HM235728; HM235740; HM235752; HM235764; *GDA55672*; Morocco, Bab Taza; GDA. ***E. semperflorens*** Wettst.; Esem01; HM235729; HM235741; HM235753; HM235765; *GDA56846*; Morocco, Essaouira; GDA. ***E. semperflorens*** Wettst.; Esem02; HM235730; HM235742; HM235754; HM235766; *GDA56847*; Morocco, Essaouira; GDA. ***E. semperflorens*** Wettst.; Esem03; HM235731; HM235743; HM235755; HM235767; Morocco, Essaouira. ***E. wilczekianum*** Braun.-Blanq. & Marie; Ewi01; HM235732; HM235744; HM235756; HM235768; *GDA56844*; Morocco, Ifrane; GDA. ***E. wilczekianum***; Ewi02; HM235733; HM235745; HM235757; HM235769; *GDA56845*; Morocco, Ifrane; GDA. ***E. wilczekianum***; Ewi03; HM235734; HM235746; HM235758; HM235770; Morocco, Ifrane.

APPENDIX 4. Variation for among-population means in the quantitative morphological traits (mean ± SE).

	Atlas populations		Rif populations	
Trait	Ene01 (N = 15)	Ene02 (N = 15)	Eri01 (N = 15)	Eri02 (N = 15)
Number of stems	8.43 ± 1.66	10.17 ± 1.11	6.11 ± 1.40	3.14 ± 1.10
Plant height (cm)	20.66 ± 0.86	19.67 ± 1.29	15.44 ± 0.51	21.77 ± 1.65
Leaf length (mm)	16.16 ± 1.56	12.66 ± 1.91	20.56 ± 2.22	23.45 ± 1.69
Leaf width (mm)	0.97 ± 0.05	0.71 ± 0.06	1.19 ± 0.13	1.25 ± 0.12
Number of flowers	32.07 ± 6.51	57.75 ± 8.18	42.22 ± 9.70	24.43 ± 7.30
Sepal length (mm)	8.11 ± 0.16	7.21 ± 0.16	7.96 ± 0.26	9.27 ± 0.28
Petal length (mm)	14.11 ± 0.41	13.58 ± 0.49	14.58 ± 0.38	15.63 ± 0.45
Petal width (mm)	2.96 ± 0.12	2.96 ± 0.20	3.88 ± 0.20	3.52 ± 0.28
Filament length (mm)	9.14 ± 0.26	8.82 ± 0.22	9.25 ± 0.22	9.58 ± 0.20
Number of fruits	28.93 ± 6.04	18.08 ± 3.05	14.89 ± 4.11	11 ± 3.44
Length of fruit pedicel (mm)	2.92 ± 0.10	2.63 ± 0.18	3.42 ± 0.28	3.67 ± 0.25
Fruit length (mm)	17.37 ± 1.37	11.14 ± 0.38	14.09 ± 0.95	23.13 ± 3.03
Fruit width (mm)	0.65 ± 0.03	0.45 ± 0.03	0.56 ± 0.03	0.77 ± 0.04