

Hybridization as a biodiversity driver: The case of *Veronica* × *gundisalvi*

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Abstract. Hybridization is an important mechanism in plant evolution, which contributes to the adaptability and biological diversity of species in fundamental ways. Based on morphological data, *Veronica* × *gundisalvi* Sennen (*Veronica orsiniana* × *Veronica tenuifolia* subsp. *tenuifolia*) is an Iberian endemic taxon of presumably polytopic hybrid origin restricted to five localities in Catalonia, where the putative parental species grow in sympatry. In this study, species distribution models were developed for the putative parental species to seek potential new localities where active hybridization could be taking place. As a result, a new location of this nothotaxon in Zaragoza is provided, along with a chromosome count and ploidy level estimations. The data presented here further support *Veronica* × *gundisalvi* as a homoploid hybrid taxon that occurs in non-altered habitats. In contrast to the traditional view of hybridization as deleterious for the conservation of biodiversity, it does not always represent a problem in this regard. Hybridization is a complex evolutionary force that requires case-specific evaluation. Given that biodiversity loss is one of the main contemporary challenges, it is important to consider the creative nature of hybridization, a widespread evolutionary mechanism able to produce novel diversity.

Keywords: Biodiversity; chromosome count; conservation; contact zones; flow cytometry; species distribution models.

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Introduction

Natural hybridization has been usually associated with areas where previously isolated lineages come into contact and mate, producing mixed offspring (Barton & Hewitt, 1989). Thus, they have been mostly viewed as ‘evolutionary noise’ or ‘blind alleys’ (Stebbins, 1950; Wagner, 1970; Arrigo & Barker, 2012). However, further studies have continuously revealed that natural hybridization is a driver of biodiversity and adaptation, being involved in the evolution of many organisms (Abbott *et al.*, 2013). Hybrid genotypes are able to occupy novel environments allowing species to expand their distribution ranges (e.g., Rieseberg *et al.*, 2003). Hybridization may enhance the adaptive potential of a species, a fact that may be deeply involved in contributing to avoid extinction risk (Chan *et al.*, 2019). Hybridization can be defined as a new combination of alleles resulting from the genomic union, which may result in novel phenotypes at the biochemical, physiological or morphological levels. In this way, hybridization may also be responsible for entirely novel evolutionary paths (Soltis, 2013). Eventually, hybridization can directly result in the formation of new species of distinct or the same ploidy level, the latter case being called homoploid hybridization. Homoploid

hybridization has been described in a great variety of organisms, and it mostly refers to diploid hybrids (Nieto-Feliner *et al.*, 2017).

Interspecific gene flow has been typically seen as a risk in conserving biodiversity, especially because endangered species may come in contact and hybridize with more common and widespread taxa (Cozzolino *et al.*, 2006). Accordingly, current conservation policies tend to disregard hybrids, hybrid zones and hybridizing species. This general negative perception is in contrast with the recognition of the historical importance of hybridization as a creative force in evolution (Abbott *et al.*, 2013). Therefore, some authors have claimed that the biological entities resulting from natural genetic admixture—which do not harm the conservation efforts on other taxa—require explicit taxonomic recognition and should not be *a priori* excluded from conservation planning (e.g., Stronen & Paquet, 2013).

Veronica subsect. *Pentasepalae* is a diploid-polyploid complex in which hybridization and polyploidization have played a major role (Rojas-Andrés *et al.*, 2015; Padilla-García *et al.*, 2018). These species are very closely related due to the recent origin of the group (mean crown age 2.8 Mya., Meudt *et al.*, 2015). Although polyploidy (allopolyploidy) seems to be the most common evolutionary mechanism within the subsection, a few

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putative homoploid hybrid populations have also been reported. According to field observations, hybridization is probably occurring in restricted areas and between certain taxa at present: *V. crinita* Kit. and *V. turrilliana* Stoj. & Stef. (European Turkey), *V. austriaca* subsp. *jacquinii* (Baumg.) Watzl and *V. aff. kindlii* Adamović (Western Balkans), and between *V. orsiniana* Ten. and *V. tenuifolia* Asso subsp. *tenuifolia* (Spain) (Rojas-Andrés & Martínez-Ortega, 2016).

Several authors provided combinations at different taxonomic ranks for these morphologically intermediate individuals between *V. orsiniana* and *V. tenuifolia* [i.e. *V. teucrium* nothovar. *marcetii* Pau, *V. × gundisalvi* Sennen, *V. × llenasii* Font Quer, *V. austriaca* nothosubsp. *gundisalvii* (Sennen) O. Bòlos & Vigo; among others]. The first formal description of these intermediate specimens at the species level was proposed by Sennen (1930) under the name *V. × gundisalvi* Sennen. The morphological characters of *V. × gundisalvi*, are intermediate between those displayed by the putative parental species (Figure 1) and shared by all known populations, all throughout its spatial distribution, which is sympatric to that of *V. orsiniana* and *V. tenuifolia* (Martínez-Ortega *et al.*, 2004; Rojas-Andrés & Martínez-Ortega, 2016). All this information has contributed to hypothesize the hybrid origin of this taxon, which is nowadays usually treated at the specific rank (Martínez-Ortega *et al.*, 2009). Although there is a complete lack of conclusive data (e.g., genetic, reproductive, etc.), multiple sources of evidence suggest that gene flow and introgression of the hybrid with the parental species is possible and thus that the three “taxonomic entities” involved are most probably inter-fertile (i.e., leaf morphology—that is the most powerful discriminant character among the taxa involved— displayed by the hybrid is a “mosaic of intermediate character states” among those displayed by the putative parents), there exist close phylogenetic

proximity between the parental species, and the hybrid is a homoploid. In this situation, for simplicity, we will adopt along with the text the nothospecific rank for this hybrid entity. *Veronica × gundisalvi* is distributed in Catalonia, where five populations have been reported so far. Some of these populations are known since the 1990’s and have been found in subsequent field campaigns (e.g., Martínez-Ortega *et al.*, 2004). Thus, they seem to be stabilized in their natural habitats. *Veronica* subsect. *Pentasepalae* comprises herbs with branched rhizome that produces numerous adventitious roots and new vegetative shoots (Watzl, 1910), which indicates that vegetative propagation is common. Although there is no information regarding seed fertility in *V. × gundisalvi*, their populations might be maintained, at least, by vegetative propagation. *Veronica × gundisalvi* constitutes an ideal study system for hybrid speciation. Contemporary methodologies such as species distribution modeling (SDMs) are widely used to gain insights into ecological or evolutionary drivers and predict habitat suitability across large scales by relating environmental variables to species records (Elith & Leathwick, 2009). They are increasingly being used to address a diverse range of applied and theoretical questions.

The main objective of the present study is to seek potential new localities of the putative hybrid species *V. × gundisalvi* in other contact zones. For this, a combined approach comprising current potential distributions, chromosome counts, and ploidy level estimations is applied in order to: (1) explore the geographic distribution of the putative parental species *V. orsiniana* and *V. tenuifolia* subsp. *tenuifolia* through SDMs to detect new possible hybrid zones, and (2) determine whether the ploidy of the plants that grow in these locations is congruent with a case of homoploid hybridization (i.e., diploid).



Figure 1. Leaf morphology (central segment of the stem). Left to right: *Veronica orsiniana*, *Veronica × gundisalvi* and *Veronica tenuifolia* subsp. *tenuifolia*.

Material and Methods

Species distribution models (SDMs)

Species distribution models were developed in order to detect possible locations where the putative parental species may occur in sympatry outside the known distribution range of *V. × gundisalvi*. For this, only confirmed presence records of the two putative parental species were considered for the analyses (Table S.1). The 19 environmental layers of the WorldClim version 1.4 dataset (Hijmans *et al.*, 2005) were initially considered (Table S.2). Values were extracted from the layers in the occurrences with the function ‘extract’ of the ‘raster’ package (Hijmans, 2019). Geographical data (altitude, latitude and longitude) were directly obtained from GPS. The number of variables was reduced to those that were not correlated. The final set of variables was selected after applying the variance inflation factor values through the ‘vif’ function of the ‘HH’ package (Heiberger, 2017) to test for the absence of multicollinearity. The feature selection process is detailed in Supplementary Information, Methods S1. Multiple scenarios were evaluated using the R package ‘ENMeval’ (Muscarella *et al.*, 2014), which implements the Maximum Entropy algorithm (Phillips *et al.*, 2006). The models were run with the feature classes L, Q, H, LQ and LQH (where L = linear, Q = quadratic, H = hinge) and a regularization multiplier (rm) from 0.5 to 3 by 0.5. The selected methods were random k-fold with five replicates for *V. orsiniana* and the leave-one-out strategy (jackknife) in the case of *V. tenuifolia* subsp. *tenuifolia* to compensate for the low number of presence records (Pearson *et al.*, 2007). The area under the curve (AUC) and the Akaike information criterion corrected for small sample sizes (AICc) were used to evaluate the models. Following these criteria, the model showing the lowest AICc and the best AUC was selected. AUC above 0.75 was considered potentially useful, 0.80–0.90 good, and 0.90–1.0 excellent (Elith, 2002). To identify areas of possible contact zone, selected models were transformed to presence/absence. For this, cells with values over 0.5 were considered as suitable for the species.

Chromosome Counts (CHN) & Flow Cytometry (FCM)

Plant material from a newly encountered population of *V. × gundisalvi* (see results section) was used to perform karyological studies and flow cytometry analyses. For chromosome counts several floral buds at different stages from two individuals were used to obtain material from anthers and gynoecia containing cells in mitotic metaphase. This material was fixed in 3:1 absolute ethanol-glacial acetic acid and stained in 2% acetic orcein (Cour, 1954) for cytological investigations.

For the ploidy level estimations by FCM, silica-gel-dried leaves were used. Samples were prepared following the procedure described in Rojas-Andrés *et al.* (2015) using propidium iodide as staining. Leaf samples of *Raphanus sativus* (Doležel *et al.*, 1992)

were used as internal reference standard. DNA ploidy level estimations were made using a CyFlow Space (Partec GmbH, Münster, Germany) equipped with a 532 nm solid-state laser. Results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany). For each individual, the ratio of the G_0/G_1 peak positions of samples and internal standards were recorded. The coefficient of variation (CV) was calculated for each sample and the standard. The data were suitable for ploidy level and genome size estimation because the CV values of the samples did not exceed the 5% threshold.

Ploidy level estimations and 1C-values of the putative parental species were obtained from Rojas-Andrés *et al.* (2020), considering only those individuals whose CV values did not exceed the 5% threshold.

Results

Species distribution models (SDMs)

The models corresponding to potential current distributions of the species showed high predictive accuracy (AUC \geq 0.90), with AUC values of 0.94 and 0.93 for *V. orsiniana* and *V. tenuifolia* subsp. *tenuifolia*, respectively. Both models showed the lowest AICc values. For *V. orsiniana* the model selected was H (rm = 1), while for *V. tenuifolia* subsp. *tenuifolia* was LQH (rm = 1.5).

After removing the correlated variables and check the absence of multicollinearity, the environmental features finally selected were: bio4 (temperature seasonality), bio13 (precipitation of wettest month), and bio18 (precipitation of warmest quarter). From these, bio18 showed the highest explanative power in both cases (relative variable contribution: 86.9% and 67.15% for *V. orsiniana* and *V. tenuifolia* subsp. *tenuifolia*, respectively). The predicted potential current distributions of the species are displayed in Figures 2a and 2b. The currently known distribution areas of the parental species (Rojas-Andrés & Martínez-Ortega, 2016) mostly match with those predicted by the models. According to the potential distributions, *V. orsiniana* most suitable areas are in the north of Catalonia, Aragon, and Navarre, and southwards on the border of Teruel with Castellon. The model also indicates potentially suitable areas in northern Castile and Leon and some scattered locations in La Rioja, although neither the hybrid nor any parentals have ever been found there. For *V. tenuifolia* subsp. *tenuifolia* the distribution is mainly restricted to the north of Catalonia and Aragon. Locations with lower suitability values compared to the previously mentioned ones are found in Teruel, Castellon, Navarre, Basque Autonomous Community, and La Rioja.

The plausible contact areas between the species are shown in Figure 2c. Catalonia is still the region where the hybrid is more likely to occur. However, it may be possible to find established populations in other locations: The Pyrenees and pre-Pyrenees, Aragon, Navarre, southern locations in the Basque Autonomous Community, and eastern areas of Teruel.

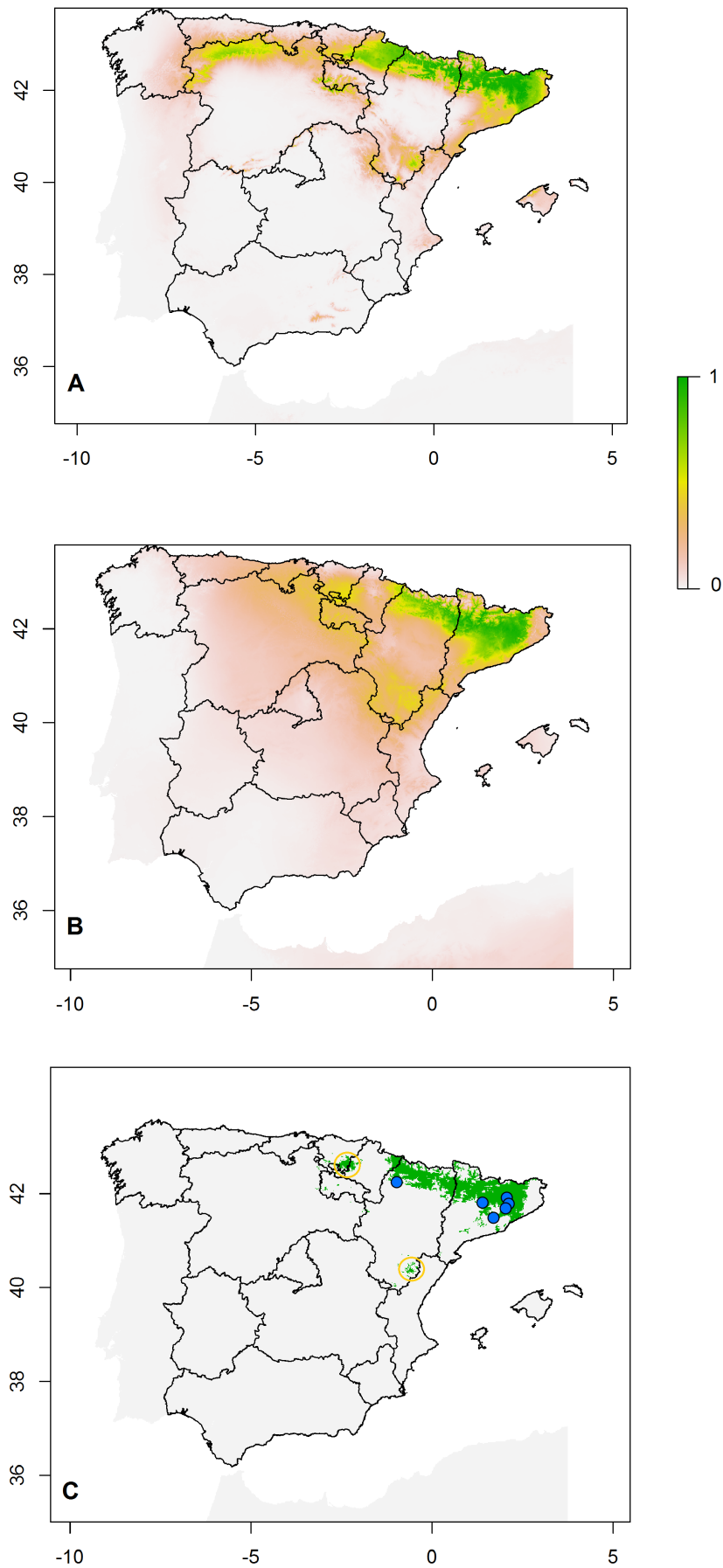


Figure 2. Predicted current environmental suitability for *V. orsiniana* (A), *V. tenuifolia* subsp. *tenuifolia* (B), and possible contact areas between them (C). The colors of the pixels represent their predicted suitability (green: maximum suitability; yellow: medium suitability; white: not suitable areas). Blue points represent known locations of *V. × gundisalvi*. Orange circles correspond to suggested locations to search for new hybrid zones.

New location for *V. × gundisalvi*, CHN & FCM

A new location of *V. × gundisalvi* was found 240 km away of the known distribution range. This location is among the places initially proposed as potential contact areas where the putative hybrid may occur. The information on this new location in the province of Zaragoza is provided here for the first time:

Veronica × gundisalvi Sennen. Spain, Zaragoza, Orés (42.276°, -0.992°; WGS84). Altitude: 562 m. Phenology:

flower/fruit. Date: 28-V-2016. Collectors: D. Gutiérrez-Larruscain and N. López-González. Voucher deposited at herbarium SALA (SALA160036) (Acronym according to Thiers (2020, continuously updated)).

The results based on both CHN ($2x = 16$; Figure 3) and FCM (Table 1) confirm that the *V. × gundisalvi* recently found population is diploid, which is in agreement with the available previous information (Table 2). As the population is composed of a low number of individuals, only material of two of them were collected.

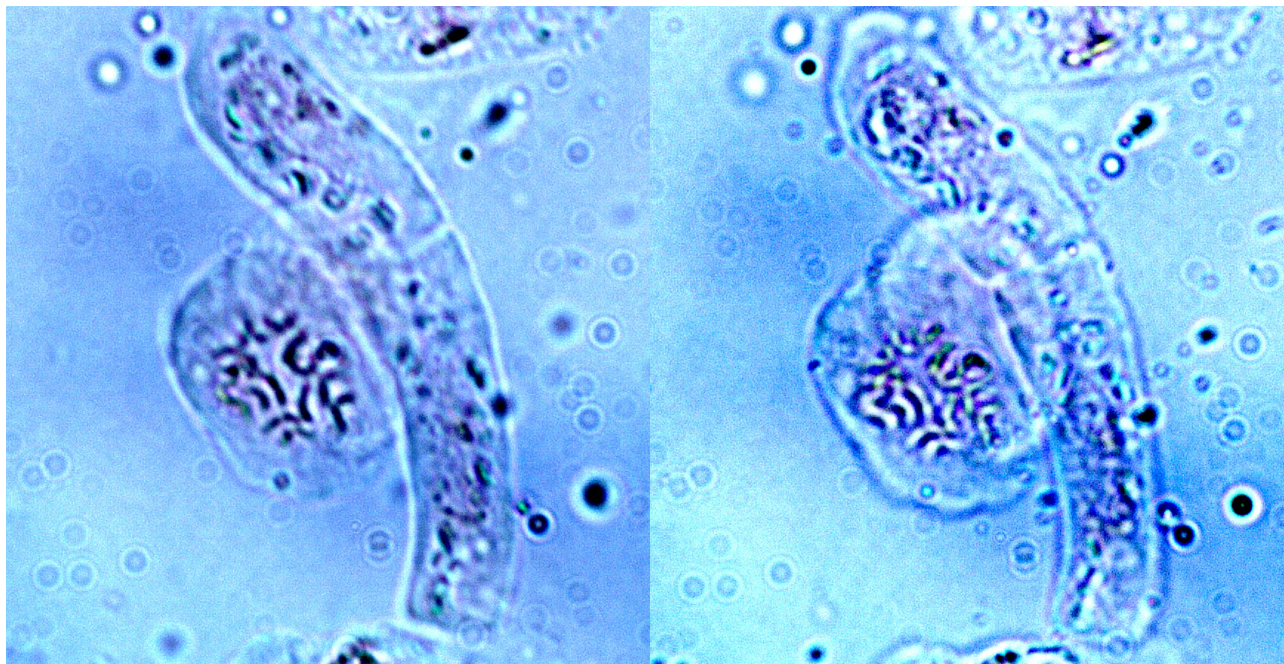


Figure 3. Mitotic metaphase. *Veronica × gundisalvi*; $2n = 16$.

Table 1. Information on ploidy level estimations. The asterisks indicate pictograms.

Species (indiv.)	1C (pg*)	Standard 1C-value (pg*)	G1 Sample	CV Sample	G1 Standard	CV Standard
<i>V. × gundisalvi</i> (1)	0.95	<i>R. sativus</i> (0.555)	178.42	3.93	104.82	4.01
<i>V. × gundisalvi</i> (2)	0.95	<i>R. sativus</i> (0.555)	179.28	3.64	105.22	3.92

Discussion

The present study brings new evidence to report *V. × gundisalvi* as a hybrid entity, in agreement with Sennen (1930) and Martínez-Ortega *et al.* (2004). Both CHN and FCM results confirm this taxon as diploid (Table 1, Figure 3). Nuclear DNA contents (1C-values) obtained here (0.95 pg for both individuals; Table 1) are intermediate between the 1C-values of the parental species (0.90 pg for *V. orsiniana* and 0.99 pg for *V. tenuifolia* subsp. *tenuifolia*; Table 2). Intermediate DNA contents between those of the parental species have been observed in sympatric sites of other homoploid hybridizing species (e.g., Baack *et al.*, 2005; Agudo *et al.*, 2019), supporting the possibility that current hybridization processes could be taking place on these locations. Individuals

of *V. orsiniana* appear a few meters away from the *V. × gundisalvi* individuals in the population found in Zaragoza, and *V. tenuifolia* subsp. *tenuifolia* populations has been found in geographic closeness. There is a wide suitable area for *V. tenuifolia* subsp. *tenuifolia* around the new location of *V. × gundisalvi* which has not been explored in detail yet. Fluorescence *in situ* hybridization experiments including the three taxa or microsatellite genotyping comparing *V. × gundisalvi* with the putative parental species and/or F_1 synthetic hybrids (i.e. genetic evidence of hybridization) would definitely confirm the status of *V. × gundisalvi* as a homoploid hybrid species. If this is confirmed, *V. × gundisalvi* would represent a hybrid biological entity with an extremely narrow distribution, which will require specific conservation strategies. However, protection is generally denied

for hybrids in conservation policies (Jackiw *et al.*, 2015; von Holdt *et al.*, 2018). The rigidity of the biological species concept (Marques *et al.*, 2018), the extinction or displacement of parental species—and the consequently threat in the case of endangered species—, or the relation of hybrids with human disturbed habitats and invasive species (Cozzolino *et al.*, 2006) could represent some of the causes why natural hybridization has been considered deleterious. In contrast, *V. × gundisalvi* occurs in natural habitats through homoploid hybridization and does not displace any of the parental

taxa: *V. tenuifolia* subsp. *tenuifolia* is an endemism but it is not endangered, and *V. orsiniana* extends its range across southern parts of France to central areas of Italy (Rojas-Andrés & Martínez-Ortega, 2016). In addition, one of the possible hybridization areas determined by SDMs and where the presence of the parental species has been confirmed—Navarre, near the contact with the Basque Autonomous Community and La Rioja (Figure 2c)—, coincides with an area that has been recognized as a hotspot for hybridization and polyploidization (Marques *et al.*, 2018).

Table 2. Summary of the previous information available on ploidy level estimations and chromosome counts of *V. × gundisalvi*, *V. orsiniana* and *V. tenuifolia* subsp. *tenuifolia*. a, three to five individuals per population were included in the analyses; b, only values that did not exceed the 5% threshold were considered; pg, picograms.

Species	Number of populations ^a	Method	Number of chromosomes	Average 1C-value ^b (pg)	Reference
<i>V. × gundisalvi</i>	5	CHN	$2n \sim 2x \sim 16$	-	Martínez-Ortega <i>et al.</i> (2004)
<i>V. orsiniana</i>	14	FCM	-	0.90	Rojas-Andrés <i>et al.</i> (2020)
<i>V. tenuifolia</i> subsp. <i>tenuifolia</i>	2	FCM	-	1.00	Rojas-Andrés <i>et al.</i> (2020)
<i>V. orsiniana</i>	11	CHN	$2n \sim 2x \sim 16$	-	Delgado <i>et al.</i> (2018)
<i>V. tenuifolia</i> subsp. <i>tenuifolia</i>	3	FCM	-	0.98	Delgado <i>et al.</i> (2018)

SDMs represent a powerful tool to learn about current species distributions and a great help for several conservation-related tasks such as determining searching areas—as the present case—, establishing priority conservation zones, predicting the effects of climate change on species distribution, etc. (for a revision see Engler *et al.*, 2017).

The role of hybridization in nature goes further beyond an invasion of the genome (Mallet, 2005). Natural hybridization neither always occurs in human-altered habitats nor necessarily involves rare and/or threatened taxa as parental species. Despite this bad reputation, it is important to remember that hybridization is a complex evolutionary force widespread in nature. It is true that in some cases, it can go against conservation efforts (Wolf *et al.*, 2001), but that should not be considered the unique possible outcome. There are great examples of the role of hybridization as a driver of biodiversity, since it produces new genetic combinations (Seehausen, 2003). An increasing number of studies demonstrate that hybridization is responsible for many well-known adaptive radiations, such as these of the cichlid fishes, the silversword alliance on Hawaii, or Darwin's finches (Marques *et al.*, 2019 and references therein). Likewise, homoploid hybridization is probably behind the formation of the species-rich polyploid complex *Leucanthemum* (Wagner *et al.*, 2019). There are also proposals to take advantage of hybridization for conservation purposes: van Oppen *et al.* (2015) suggest adding genetic variation to natural populations via

human-assisted interventions to avoid the world's coral reefs extinction.

The negative perception of hybridization changes when the conservation focus is on preserving the adaptive potential of a species instead of preserving the species in its original state (Chan *et al.*, 2019). Natural hybridization may represent an advantage rather than a threat to biodiversity.

We are facing a scenario of climatic change and biodiversity loss with little resources for conservation. Thus, conservation priorities should focus mostly on protecting areas where we can still act to preserve ecological and evolutionary processes such as hybridization (Cozzolino *et al.*, 2006). Conservation strategies involving practitioners have proven to be formulas of great success (e.g., initiatives as “*Adopta una planta*”; <http://www.liferesecom.ipe.csic.es/index2.php>). These kinds of programs can be implemented by combining the involvement of the local population and the selection of areas of evolutionary interest.

Conclusions

The use of SDMs enabled the finding of a new population of *Veronica × gundisalvi* in the province of Zaragoza (Spain). The data provided here for this new population further support *Veronica × gundisalvi* as a homoploid hybrid species occurring in non-altered habitats. The loss of biodiversity is one of the main problems at present and it becomes necessary to think about the creative

nature of hybridization as a process able to produce novel biodiversity. Thus, conservation policies should take into account the contribution of hybridization to the adaptability and diversification of species, which is of key importance for the maintenance of life in our planet.

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