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Evolutionary trends in the distylous genus *Pulmonaria* (Boraginaceae): Evidence of ancient hybridization and current interspecific gene flow ☆

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ABSTRACT

The distylous genus *Pulmonaria* contains approximately 18 species that are widely distributed across Eurasia. Previous studies have shown that species delimitation in the genus is problematic, but have not yet explored the evolutionary history of the genus. Premating reproductive barriers between European species appear to be weak, as several species have strongly overlapping distribution areas, flower at the same time and share the same pollinators, suggesting that hybridization may have contributed to the evolutionary history of *Pulmonaria*. To test this hypothesis, phylogenetic analyses of nuclear ITS and plastid data (*rps16*, *trnH-psbA*, *rpl16*) from 48 allopatric and four sympatric populations were performed to (1) provide a molecular phylogeny for nine of the most common *Pulmonaria* species in Europe, (2) detect current and ancient hybridization events, and (3) assess the contribution of hybridization versus incomplete lineage sorting to the inferred phylogenetic patterns. Our results showed that gene trees displayed widespread, strongly supported incongruence associated with the conflicting position of hybrid samples rather than incomplete lineage sorting. Evidence was found of different degrees of hybridization, ranging from current interspecific gene flow at secondary contact zones to introgression at the population level and at least one event of hybrid speciation. Overall, these results suggest that hybridization and introgression were – and could still be – important processes affecting speciation in the genus *Pulmonaria*.

1. Introduction

Hybridization has long been acknowledged to be important in the process of speciation and is known to result in varied outcomes with respect to species formation (reviewed in Mallet, 2007; Abbott et al., 2013). Interspecific gene flow at secondary contact zones may slow down or even cause lineage convergence due to introgression and occurs more readily among related species as a result of the gradual increase of reproductive isolation with genetic divergence (Coyne and Orr, 1989, 1997; Moyle et al., 2004; Scopece et al., 2007). On the other hand, hybridization may promote rapid reproductive isolation of sympatric species due to selection against the formation of unfit hybrid offspring ('reinforcement'), thereby accelerating the process of speciation (Servedio and Noor, 2003). Furthermore, new favorable gene combinations resulting from hybridization may give rise to new species, which can become instantaneously reproductively isolated from the parental species (Vereecken et al., 2010). Hybridization is considered a regular event in species biology with 25% of the plant species known to hybridize with at least one other species (Mallet, 2007).

The genus *Pulmonaria* (Boraginaceae) has been of interest for centuries to botanists worldwide because of its ornamental and medicinal properties (Hewitt, 1994; Bennett, 2003). In addition, *Pulmonaria* is known for its complex taxonomy and problematic delimitation of species (Sauer, 1975; Bolliger, 1982) as well as its characteristic distylous breeding system throughout the whole genus (Darwin, 1877; Olesen, 1979; Richards and Mitchell, 1990; Champluvier and Jacquemart, 1999; Meeus et al., 2012a,b). Based on a previous molecular phylogeny, *Pulmonaria* is placed within the generic complex *Nonea*/*Elizaldia*/*Pulmonaria*/*Paraskevia* (Boraginaceae, Boragineae) (Selvi et al., 2006). *Pulmonaria* separated from a common Tertiary ancestor with *Paraskevia cesatiana*, a tetraploid ($2n = 28$) Greek endemic characterized by a non-rhizomatous root system, the absence of heterostyly and the prefloral development of foliage leaves, traits that differentiate this species from the

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typical *Pulmonaria* species (Selvi et al., 2006; for the most recent placement of *Pulmonaria* within the Boraginaceae, see Cohen, 2013).

Morphologically, *Pulmonaria* species are fairly similar in having pink-blue[1] actinomorphic tube-shaped flowers and hairy leaves (Fig. 1). As a result, they were often considered as varieties of the same species rather than as separate species (Darwin, 1877). According to the most recent monographic treatment of the genus by Bolliger (1982), the genus *Pulmonaria* comprises 18 species (8–11 subspecies), of which eight occur in Western Europe. *Pulmonaria obscura*, *P. officinalis*, *P. longifolia* and *P. angustifolia* are the most common species in Western Europe and have largely overlapping distribution areas, which together cover almost the entire range of *Pulmonaria* (Fig. 2). Although the majority of species occurs most often in shaded woodland habitat (e.g. *P. officinalis*, *P. obscura*), some species (e.g. *P. longifolia*) prefer dry and sunny habitats and are, therefore, often found in grasslands (Hill et al., 2004).

Although the genus *Pulmonaria* is morphologically uniform, chromosome numbers vary widely among and even within *Pulmonaria* species and subspecies, having a basic haploid chromosome number of $n = x = 7$. Therefore, chromosome counts have been intensively used for species identification and have provided evidence of polyploidization events and the occurrence of aneuploid species (i.e. having incomplete sets of or deviations from the basic chromosome number) within the genus (Sauer, 1975; Bolliger, 1982). Chromosome numbers ($2n$) range from 14 (present in at least 40% of the *Pulmonaria* species) to 38 with a total of 10 different cytotypes (Sauer, 1975). *Pulmonaria* contains diploids ($2n = 2x = 14$), tetraploids ($2n = 4x = 28$) and a series of aneuploids ($2n = 16, 18, 20, 22, 24, 26, 30, 38$) (Sauer, 1975; Bolliger, 1982).

Little is known about the strength of the different isolating barriers in *Pulmonaria*, however, the apparent lack of premating barriers (i.e. strong overlap in geographic distribution, flowering synchrony, similar pollinator preference) and potential strong postmating barriers resulting from the high variation in chromosome numbers within the genus has made that the extent to which *Pulmonaria* species hybridize and how this process contributed to speciation within the genus has been subject to debate during the last century (Gams, 1927; Merxmüller and Sauer, 1972). Moreover, historical records report opposite results regarding compatibility among the species and the incidence of hybridization. For example, Darwin (1877) reported a failure of seed set after pollinating *Pulmonaria longifolia* populations from the Isle of Wight (UK) with pollen from *P. officinalis*. In horticulture, however, *Pulmonaria* is known to hybridize freely among species, resulting in popular varieties such as *Pulmonaria* 'Mawson's Blue' and *Pulmonaria* 'Sissinghurst White' (Bennett, 2003). These popular ornamental hybrids are, in turn, used to generate new varieties implying that hybrids are not only viable, but also able to reproduce sexually (Bennett, 2003). Moreover, few interspecific crosses performed by Bolliger (1982) suggests that first-generation hybrids show regular bivalent pairing and high pollen viability (60–89%) at meiosis both for intra- and interploidy crosses. Nevertheless, taxonomists agree on the species status of these common *Pulmonaria* species based on their morphological traits and chromosome numbers (Sauer, 1975; Bolliger, 1982).

Heretofore, only few attempts have been made to reconstruct a molecular phylogeny of *Pulmonaria* (Kirchner, 2004) and to test specific hypotheses about the role of hybridization in affecting the complex evolutionary history of the genus. Reconstruction of phylogenetic relationships using multiple genetic markers, however, is problematic as hybridization is a major cause of topological incongruence between gene trees (McBreen and Lockhart, 2006). Horizontal gene transfer between closely or more distantly related species often results in a strong conflict between gene trees with the traditional bifurcating (hierarchical) representation of species diversification and is therefore best presented by a reticulation network (Hennig, 1966; Legendre and Makarenkov, 2002; Huson et al., 2005; Hegarty and Hiscock, 2005; McBreen and Lockhart, 2006). However, studying the incongruence between gene trees of hybridizing taxa offers an opportunity to detect hybrid speciation (Sang and Zhong, 2000). Moreover, by comparing differently inherited markers – e.g. nuclear (biparentally inherited) and plastid genomes (maternally inherited in case of *Pulmonaria*) – it is possible to identify the paternal and maternal species involved in current and historical hybridization events (Linder and Rieseberg, 2004). Several tests have been proposed to assess the statistical significance of phylogenetic incongruences (Farris et al., 1995; Shimodaira and Hasegawa, 1999; Shimodaira, 2002) and significant topological differences are increasingly attributed to interspecific hybridization (McBreen and Lockhart, 2006 and references therein).

However, confounding population genetic processes such as lineage sorting (i.e. the stochastic sorting of alleles following divergence from a polymorphic ancestor) might mislead inference of the real contribution of hybridization to the observed pattern of gene tree incongruence (Linder and Rieseberg, 2004; Kubatko, 2009; de Villiers et al., 2013) and is especially common among closely related species where lineage sorting has not yet been completed, leading to non-monophyletic species assemblages (e.g. *Primula*; Schmidt-Lebuhn et al., 2012). Incomplete lineage sorting is especially important when the effective population size of a given lineage is large with respect to the time elapsed since divergence, so that genetic drift is unlikely to result in fixed alleles subsequent to divergence (Rosenberg and Nordborg, 2002). Since uniparentally inherited plastids have an effective population size which is smaller than that of biparentally inherited nuclear markers, the rate of lineage sorting is expected to be faster in plastids (Palumbi et al., 2001; Hedrick, 2007). Deviations from this expectation could, therefore, be caused by hybridization events (Chan and Levin, 2005). A third approach to detect hybridization between a group of species which has been increasingly used in several studies concerns the analysis of sequences from multiple gene families (e.g. ITS) to look for different intra-individual copies that hold information about the hybrid's parentage to get a more direct assessment of hybrid evolution of which patterns are not subjected to incomplete lineage sorting (e.g. Koch et al., 2003; Feliner et al., 2004; Hodač et al., 2014).

In the present study, we investigated whether a statistically significant phylogenetic conflict between nuclear and plastid genomes was apparent in the genus *Pulmonaria*, providing support that hybridization has played a significant role in intrageneric speciation processes. For the specific detection of potential hybrid ("conflicting") populations and the assessment of ongoing gene flow, morphologically intermediate individuals from sympatric populations were included in the analysis and ITS polymorphisms were investigated. Furthermore, the Genealogical Sorting Index (GSI) was calculated to infer the contribution of incomplete lineage sorting versus hybridization to the observed phylogenetic pattern.

2. Materials and methods

2.1. Sampling and species identification

Leaf samples were collected across seven European countries (Belgium, France, England, Germany, Czech Republic, Italy, Estonia) during spring 2013 from 48 allopatric populations of nine species

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[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.