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RESEARCH ARTICLE

Are *Melilotus albus* and *M. officinalis* conspecific?

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Abstract The principal economic species of the genus *Melilotus* are white sweet-clover (*Melilotus albus*) and the extremely similar yellow sweet-clover (*M. officinalis*). Although they are widely recognized as distinct species, some influential references in North America reduce the former to a subspecific rank or even merely a conspecific synonym of the latter. Given their importance and the large numbers of germplasm collections, the doubt needs to be resolved. This review of relevant published evidence finds that in addition to the difference in floral colour, the traditional segregation of the two as distinct species is best supported by very strong reproductive barriers as well as divergent DNA sequences in three barcoding genes. Additional but weaker confirmation of separateness is provided by studies reporting differences in external morphology, biochemistry, seed protein profiles, karyotype and DNA microsatellites.

Keywords *Melilotus albus* · *Melilotus officinalis* · *Trigonella alba* · *Trigonella officinalis* · Sweet-clover · Melilot · Conspecific · Species definition

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Introduction

Melilotus albus Medikus (white sweet-clover; Fig. 1A) is grown as a forage crop, green manure, honey plant, wildlife habitat enhancer, and roadside and revegetation cover, while also posing a widespread weed problem (Turkington et al. 1978). (The gender of *Melilotus* has often been treated as feminine, resulting in the name *M. alba.* However, under the International Code of Nomenclature (McNeill et al. 2012) the name must be treated as masculine.) The very similar *M. officinalis* (L.) Lamarck (yellow sweet-clover; Fig. 1B) is employed less frequently for the same purposes, and is more significant as an undesirable weed (Turkington et al. 1978; Van Riper and Larson 2009). In most floras and identification keys the two are distinguished by the white petals of the former and the yellow petals of the latter. In Eurasia, where there are many species of the genus, identification can be challenging, but in North America essentially all white-flowered *Melilotus* plants growing outside of cultivation would be identified as *M. albus*, and the vast majority of yellow-flowered plants as *M. officinalis* (other yellow-flowered species are rarely reported for North America; Small, in press). However, some competent North American botanists have concluded that the two are merely conspecific colour morphs that do not merit taxonomic distinction, relegating *M. albus* to synonymy (or subspecific rank) with *M. officinalis*. This taxonomy

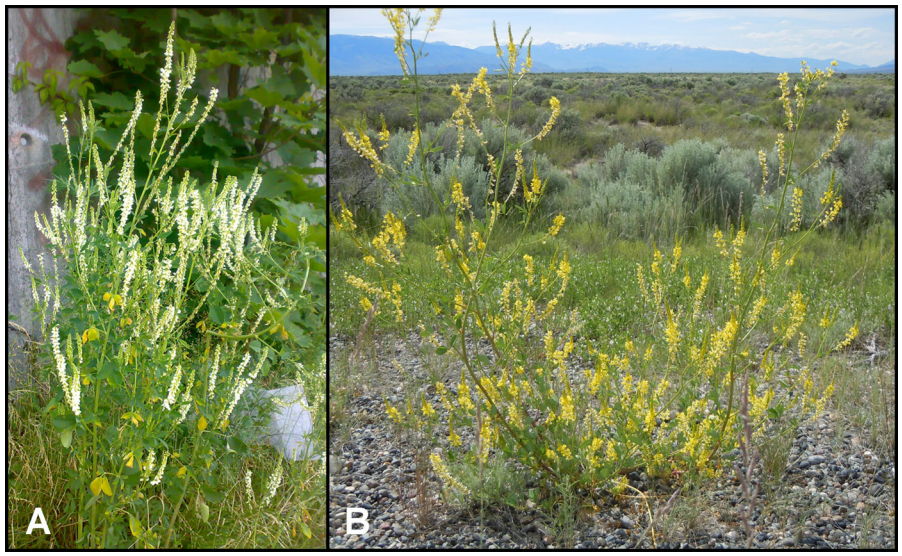

Fig. 1 Ruderal *Melilotus* plants. **A** *M. albus*. Photo by Bjoertvedt (reproduction license: CC BY SA 3.0). **B** *M. officinalis*. Photo by Matt Lavin (reproduction license: CC BY SA 2.0)

has been promulgated through a number of extensively used online Internet databases. The database of Kartesz (2015) is the most influential and the basis of several US government supported taxonomic database initiatives including the USDA PLANTS database (USDA, NRCS 2018), the Integrated Taxonomic Information System (ITIS 2017) and the BugwoodWiki website (Bugwood 2017). The Catalogue of Life website (CoL 2017) "accepts" *M. albus* both as a distinct species and as a subspecies of *M. officinalis*. The second author of this review is responsible for the treatment of *Melilotus* in the Flora of North America project (in preparation), and was requested by the editors to address the viewpoint of some taxonomists that the two are conspecific. Clearly, at least in North America, there is support for this interpretation.

The similarity of the two species was noted as long ago as 1651 by J. Bauhin (Historia Plantarum II, p. 370) who considered *M. albus* to be merely a white-blossom form of *M. officinalis*. Linnaeus, in his Species Plantarum of 1753, treated *M. albus* as a subspecific variety, designated as γ, of "*Trifolium* (*Melilotus*) *officinalis*" (see Sales and Hedge 1993 for analysis). This interpretation was also maintained by Persoon (1807) as *M. officinalis* β *albus* and later formalized as *M. officinalis* subsp. *albus* (Medikus) H. Ohashi et Tateishi (Ohashi et al. 1984). We have not found any literature which explicitly gives evidence or reasoning for this taxonomic approach. In this note, we review evidence bearing on the separation of the putative taxa.

Melilotus albus and *M. officinalis* make up the majority of the thousand or so sweet-clover germplasm accessions of the U.S. Department of Agriculture (Brenner 2005), reflecting their predominant economic importance of the 20 or so species usually recognized in the genus (Stevenson 1969), although some authors such as Smith (1927) and Suvarov (1961) accept a larger number of species.

Molecular analyses (Bena et al. 1998; Dangi et al. 2016; Wojciechowski et al. 2000; Steele and Wojciechowski 2003; Wojciechowski 2003) have indicated that at least some of the species of *Trigonella* and *Melilotus* belong to the same phylogenetic clade, and accordingly the two genera should probably be combined. Accepting this, the two species discussed here become *T. alba* (Medikus) Coulot et Rabaute and *T. officinalis* (L.) Coulot et Rabaute (Coulot and Rabaute 2013). The generic status of *Melilotus* is beyond the scope of this paper, and this nomenclature is not employed simply because it is not yet widely accepted.

Review of evidence

Morphology

Melilotus albus and *M. officinalis* are very similar, and cannot be distinguished by appearance with much certainty when in a vegetative state. Both species are quite variable, likely augmented by the selection of many cultivated forms, their distribution by humans to much of the temperate and subtropical world and subsequent evolution of local ecotypes. Isely (1954) observed that European material of *M. albus* is more polymorphic by comparison to wild and cultivated strains in North America, and since both species are indigenous to the Old World where they have differentiated for millennia, the same is probably true for *M. officinalis*. It is possible that in some countries (especially where the species have been introduced) the range of morphological variation is relatively limited, and accordingly they might be distinguished by characteristics that are not applicable to the plants in other locations. The following are the principal visual characters that have been employed for identification.

Flower colour

Flower colour has been the prime basis of distinguishing *M. albus* and *M. officinalis* for over two centuries by most botanists. Various authors in different countries have claimed that, excepting flower colour, there are other differences, but from a global perspective only flower colour has been universally, and reliably, employed as a discriminatory character. Unfortunately, herbarium specimens often do not show the colour distinction well, the flowers of both species frequently fading to a dull cream with age. One-character taxonomies are suspect, especially with flower colour, since simple allelic changes in one of the wide range of structural or functional pleiotropic genes or cis-regulatory elements may be responsible for an achromatic polymorphism (Coburn et al. 2015; Iida et al. 2004). Genetic mechanisms involved in colour polymorphism within a species versus the stabilized colour differences between divergently evolved species appear to be different in nature (Coburn et al. 2015; Wu et al. 2013), but little is known of the genetic mechanisms determining flower colour in *Melilotus*. Indeed, forms with atypical flower colour for *Melilotus* species are frequent (Schulz 1901), suggesting that phytochrome pathways may be susceptible to mutation in the genus.

Using the one confirmed hybrid plant (*M. albus* × *M. officinalis*) which he was able to obtain (see below), Kirk (1931) examined flower colour inheritance in 150 F_2 plants. He classified the results into five groups (white, dull white, light cream, dark cream and yellow). By self-pollinating plants from each of the colour groups he obtained 54 F_3 families, but the increase in flower colour shading made classification more difficult. From his analysis of the colour frequencies, Kirk (1931) concluded that three genes are involved, C_1 and C_2, which are of unequal effect in determining cream colour and W, which inhibits the effect of C_2. Although the three factor model fit his observations well, there were still lower than expected numbers of yellow flowers in many F_3 families. Clearly colour determination is not controlled by simple single-locus allelic variation, but involves genes for both pigment production and regulation of gene expression.

Fruit venation pattern

The venation areolae on the mature pods differ somewhat between the two species: the raised venation ridges tend to form an irregular reticulation on the mature fruits of *M. albus*, whereas they tend to form transverse areolae on the fruits of *M. officinalis* (Stevens and Long 1926; Townsend and Guest 1974; Fig. 2). This difference is frequently cited as a complementary diagnostic character (in addition to flower colour). Although this character is variable and somewhat difficult to assess, it does seem to be the most reliable discriminatory feature aside from flower colour.

According to Voronchikhin (1990), *M. albus* and *M. officinalis* could be clearly distinguished by the surface texture (epidermal cell sculpturing) and the anatomical structure of the testa. However, such micro-morphological features are difficult to employ and large samples have not been examined.

Flower length

Isely (1954) used flower length as a supplementary feature to separate *M. officinalis* from *M. albus*: the former allegedly having shorter flowers [3–5 (5.5) mm

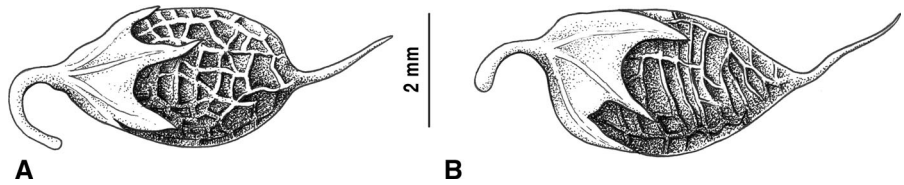

Fig. 2 Venation patterns on fruit walls of **A** *Melilotus albus* and **B** *M. officinalis*. Note that the venation ridges of the former tend to produce loops delimiting spaces (areolae) that are approximately as long as wide, whereas some areolae of the latter are noticeably transversely elongated. Drawn by J. Hsiung, based on representative specimens of the DAO herbarium

vs. 4–5 (5.5) mm]. Stevenson (1969) gave ranges for the two as 4.5–7 mm versus 4–6 mm, respectively. Measurements given by Kita (1965) also attribute larger flowers to *M. officinalis*, presenting data roughly similar to those of Stevenson (1969). The character of flower length appears to be unreliable for diagnostic purposes, at least as far as can be ascertained by the published data. The differences between the reported size ranges may be due to reliance on commercial cultivars, which tend to have restricted ranges of variation.

Relative length of alae and keel

Several keys in national floras employ (as a secondary character after flower colour) relative ala (wing) and keel lengths (longer than keel in *M. officinalis*, equal in *M. albus*). Examples of this are Chamberlain (1970) and Ali (1977). However, Isely (1954) in his key stated that the wings merely tend to be longer than the keel in *M. officinalis*. Stevenson (1969) noted "the relative lengths of keel, wing and standard, usually constant and therefore good characteristics from the taxonomist's point of view, have shown considerable variation." The character, therefore, appears to have limited utility.

Seed mottling

Because *M. albus* and *M. officinalis* are economically important, seed testing laboratories have assessed methods to distinguish their seeds (Maxon and Hurst 1983). Sunken brownish spots on the seeds have been found to be sites of water penetration in some biotypes of the two species (Stevenson 1937). Such seed mottling or spotting may have some limited discrimination value (Stevens and Long 1926; Downey et al. 1954; Musil 1963; Maxon and Hurst 1983). According to Whitcomb (1930), "The lack of uniformity in the mottling of the seeds of these two species and the absence of other well defined characteristics make it difficult for the [seed] analyst to make very definite distinctions." Kirk and Stevenson (1931) found the seed spotting characteristic to be present in both species.

Other morphological features

Stevens and Long (1926) note a few subtle differences in seed shape which do not seem to be reliable enough for taxonomic purposes, especially when seed development is constrained in two-seeded pods. Some works on seed identification suggest that the angle formed between the hypocotyl and radicle tends to be more acute in *M. albus* than *M. officinalis* (Beijerinck 1947; Martin and Barkley 1961; Musil 1963), but this character is also quite variable.

In the plants that were studied by Kita (1965), inflorescences of *M. officinalis* were longer and contained an average of 54.1 (SD = 2.6) flowers, while the two genotypes of *M. albus* he examined were considerably shorter and had 68.1 (SD = 8.3) and 68.7 (SD = 7.8) flowers per inflorescence. The inflorescences at the peak of flowering have been reported as being 8–15 times longer than broad in *M. albus*, but only about 6 times as long as broad in *M. officinalis*, and leaflets 2.5–3.5 times as long as broad versus usually no more than 2 times longer than broad, respectively, for the plants naturalized in the Great Lakes region of North America (Voss and Reznicek 2012). These distinctions might be useful in this region but may not be more widely applicable. The overlapping ranges of these quantitative character states are phenotypically variable and not particularly useful for identification.

Pollen grain size has been reported to be distinctly different between *M. albus* and *M. officinalis*, respectively 15.2–17.1 μm wide × 20.9–22.8 μm long,

versus 16.9–19.0 μm wide × 24.7–26.6 μm long (Crompton and Wojtas 1993). However, the sizes reported by Coe and Martin (1920) were considerably larger and overlapped in range: 26 × 32 μm for *M. albus* and 24 × 30 μm for *M. officinalis*.

Geography and ecology

Both *M. albus* and *M. officinalis* are indigenous to Eurasia, and have been widely introduced to other areas of the world (for maps see Hultén and Fries 1986). In many regions they grow in proximity and in the same habitats, but frequently they seem to differ somewhat in their occurrence and ecology (Turkington et al. 1978; Gucker 2009). This may be the result of introduction of local biotypes, especially of semi-domesticated forms of *M. albus*, which often produce comparatively larger and more vigorous plants in comparison with *M. officinalis*. Meyer (2005) commented "yellow-flowered sweet-clover is shorter growing, more widely branched, finer stemmed, more drought tolerant, easier to establish and better adapted to the drier regions of North Dakota than white-flowered sweet-clover." Geographical location, habitat, and ecology are not reliable guides to discriminating the two species, but may indicate significant physiological differentiation.

Several North American authors have noted that weedy populations of *M. officinalis* commence flowering about 10 days earlier than *M. albus* (Stevens and Long 1926; Voss and Reznicek 2012). Since there is a long overlapping period in flowering, the potential for cross-pollination remains.

Reproductive barriers

Melilotus species are diploid with $2n = 16$ (Sano et al. 1991), although tetraploids have been artificially created. Both *M. albus* and *M. officinalis* are primarily outcrossing (Gucker 2009), pollinated by a wide variety of common hymenoptera (Coe and Martin 1920), and, inasmuch as they are widely sympatric and often grow in mixed populations, there is enormous opportunity for natural hybridization to occur. The fact that hybrids are very rarely reported is indicative of substantial barriers to interbreeding. Several kinds of reproductive barriers among species of *Melilotus* were investigated by Sano and Kita (1978b).

There is reason to doubt most reports of natural hybrids between *M. albus* and *M. officinalis* (Stevenson and Kirk 1935; Smith 1954) since, as noted below, there is evidence of substantial inter-sterility, and there do not seem to be records of natural hybrids that are fertile. Schulz (1901) referred to a natural hybrid between *M. albus* and *M. officinalis*, but provided almost no information about it. Occasionally, others have similarly reported hybrids, but with no supporting observations. Sylven (1929) noted that among the seeds he imported from Canada two plants produced pollen that was 50%–80% sterile, in flowers that were pale yellow or yellowish-white, and he interpreted these as natural hybrids between the two species as described by Kirk (1929). However, Kirk (1929) grew the two species side by side in an open-field experiment under optimal conditions. A total of 11,400 plants were examined for possible hybrids in the following generation. Only a single plant which produced cream-coloured flowers could be confirmed as a hybrid via F_2 segregation of colour forms—a hybridization rate of < 0.00001. The artificial hybrids produced by Maekawa et al. (1991) also possessed cream-coloured flowers, possibly the only external feature, other than pollen "sterility", that is of any use in identifying natural hybrids.

There have been exhaustive attempts at artificial hybridization between *M. albus* and *M. officinalis*. Kirk (1930) and Stevenson and Kirk (1935) performed over 7000 crosses between them, producing no viable seed but only a number of abortive embryos. Both Johnson (1942) and Smith (1954) were similarly unsuccessful at crossing *M. albus* and *M. officinalis*, as was Kita (1965) in 55 crossing attempts. Greenshields (1954) found that embryos of the hybrid sometimes survived for up to 2 weeks before their erratic development ceased, with embryos of *M. albus* (pistillate) × *M. officinalis* (staminate) aborting earlier than the reciprocal cross. Lang and Gorz (1960) similarly studied the artificial generation of hybrid embryos which almost always aborted. In their extensive crossing experiments, Lang and Gorz (1960) obtained 12 hybrid seeds which germinated and grew to maturity. However, 11 of these were derived from crosses between *M. albus* 'Spanish' and *M. officinalis* P. I. 178985 (USDA accession), and they described the latter, perhaps a misidentified parent, as, "unlike known strains of *M. officinalis* in several ways." Using P. I. 178985 as the pistillate parent in

crosses with *M. albus*, Sano and Kita (1975, 1978a) were also able to generate hybrid seedlings, but these had a chlorophyll deficiency, disrupted chromosome pairing in meiosis and 51.0–67.1% pollen fertility (acetocarmine stained). Webster's (1955) hybridization experiments led to his concluding that there are very strong barriers to interbreeding between the two species, such that reciprocal crosses are usually unsuccessful or merely result in a stimulation of ovule development, but not viable seeds. Nevertheless, using embryo rescue techniques he was able to produce two mature hybrid plants, and he stated that the degree of hybridization compatibility between *M. officinalis* and *M. albus* differed somewhat depending on the particular combination of genotypes. Czigat (1966) similarly generated hybrids of the two species, but once again only with the aid of embryo culture. Shastry et al. (1960) were unable to generate hybrids between the two species, but found that one of Webster's (1955) embryo-rescue hybrids exhibited 75% pollen fertility and near-normal meiosis, from which they concluded that there were few chromosomal structural differences and attributed hybridization failure to somatoplastic factors.

Melilotus albus is not only largely incompatible with *M. officinalis*, it is also genetically isolated from some other species in the genus (*M. altissimus* Thuill., *M. dentatus* (Waldst. & Kit.) Pers., *M. tauricus* (M. Bieb.) Ser. and *M. wolgicus* Poir.) in that hybridization, when successful, results in chlorotic F_1 seedlings (Smith 1954; Lang and Gorz 1960). Other species of yellow-flowered *Melilotus* which have been successfully crossed with *M. albus* are *M. suaveolens* Ledeb. and *M. polonicus* (L.) Pall., although success sometimes varies with parental genotypes and crossing directionality (Stevenson and Kirk 1935; Johnson 1942; Smith 1954).

Chromosomes

The two species are reported to differ in chromosome (karyotype) morphology. In his cytogenetic study of the genus, Clarke (1934) reported that one pair of chromosomes in both *M. albus* and *M. officinalis* possesses satellites. He described the satellites as being larger and the satellite-bearing chromosomes as being smaller in *M. officinalis* than *M. albus*. Sano and Kita (1975, 1978a, b), Maekawa et al. 1991 and Ha (1993) observed very strong barriers to hybridization, and, from their cytogenetic studies, deduced that *M. albus* and *M. officinalis* differed in chromosome structure as a result of a large reciprocal translocation.

Chemistry

Seed testing laboratories sometimes employ a chemical test to distinguish *M. albus* and *M. officinalis* (Elekes and Elekes 1972; Maxon and Hurst 1983). By this test, a blue solution, produced by combining cupric sulfate and ammonium hydroxide to form tetraamincopper sulfate, is applied to abraded seeds soaked for several hours in water, and within 15 min the seed coats of *M. officinalis* are said to turn dark brown or black while those of *M. albus* are said to turn olive or yellowish green. Dayton (1975) found that he was able to distinguish the two species by amino acid composition of the seeds. Sixteen species of plants in seven families were screened for antibacterial and antitumor activity by Karakaş et al. (2012), including *M. albus* and *M. officinalis*, which suggested significant biochemical differences between the two *Melilotus* species. Water and ethanol extracts of *M. officinalis* showed antibacterial activity against a number of gram-negative bacteria (sometimes equal or greater than antibiotic positive controls), while *M. albus* showed no activity. Conversely, water and methanol extracts of *M. albus* showed significantly greater tumor inhibition than *M. officinalis*. None of these claimed differences have been validated by tests with large, representative samples of the species.

No species-distinguishing patterns in six isozymes (EST, IDH, LAP, PGI, PGM and POX) were seen in 15 accessions of *M. albus* and ten accessions of *M. officinalis* by Ha (1993). However, a principal component analysis of the SDS-PAGE seed protein banding (49 bands) clearly separated *M. albus* from *M. officinalis* along the second Eigen vector (Ha 1993), indicating a significant difference in the seed protein profile.

DNA sequencing

Several studies have been conducted to examine the genetic variability and differences within and between the two species *M. albus* and *M. officinalis*. A study of SSR (simple sequence repeats or microsatellites) variability in the two species from an introduced roadside population at Healy, Alaska, was conducted

by Winton et al. (2007). They reported variation in the heterozygosity and differing linkage disequilibrium patterns between the two species. The low levels of observed heterozygosity and different numbers of alleles found at six of the nine loci suggest that there is little, if any, gene flow between white and yellow-flowered plants in this single mixed population. Studies of the allelic variation at SSR loci in 18 species of *Melilotus*, found that *M. albus* and *M. officinalis* clustered distantly from one another (Wu et al. 2016; Yan et al. 2017). The published UPGMA dendrograms (based on Nei's genetic distance) of SSR alleles show both as being related, but more similar to other *Melilotus* species in subgenus *Melilotus* than to each other.

The sequences of five "barcoding" loci were published for 19 species of *Melilotus* by Wu et al. (2017). These loci included the nuclear ITS (646 bp), the chloroplast *rbc*L (754 bp) and *mat*K (714 bp), and the non-coding chloroplast *trn*H-psbA (306 bp) and *trn*L-F (451 bp). Their published sequences revealed significant differences between *M. albus* and *M. officinalis* in the sequences of ITS (1 transversion), *mat*K (two transversions) and *trn*L-F (two transversions and four indels totaling 27 bp). This represents at least five mutations involving 32 base pair positions out of a total of 2871 (1.1%). Although the intraspecific sequence variation in *Melilotus* species has not been extensively investigated, the consistent differences in the sequence data presented by Wu et al. (2017) for three genes that are frequently used for species barcoding is strongly indicative that *M. albus* and *M. officinalis* are indeed distinct species.

Phylogenetic analyses of 18 *Melilotus* species using DNA sequences from the nuclear locus ITS and the three chloroplast loci *mat*K, *rbc*L and *trn*L-F (Di et al. 2015), indicated that the white-flowered species in the genus are polyphyletic. The white flower character state appears to be apomorphic and has evolved several times in the genus. The authors conducted four maximum parsimony analyses (using sequences from ITS alone, *rbc*L alone, the three chloroplast loci together and a combination of all four loci). Although there are varying degrees of resolution in the cladograms presented, all show *M. albus* and *M. officinalis* in different clades, except for the *rbc*L analysis which placed the two species in an unresolved clade of ten. The other three trees maintained these ten species together, in somewhat different topologies, as a holophyletic clade. They also consistently placed *M. albus* and *M. officinalis* in divergent groups. In fact, based on these analyses, if *M. albus* and *M. officinalis* are to be combined and a holophyletic classification is to be maintained within *Melilotus*, all ten species identified in this clade would have to be considered conspecific.

Discussion

In the extensive interspecific breeding programs of *Melilotus* by various workers, crosses between *M. officinalis* and *M. albus* very rarely produced F_1 plants. Morphological, cytogenetic and molecular studies have all indicated a close relationship between *M. albus* and *M. officinalis*, but breeding studies have shown that there are strong physiological barriers to fertilization, seed set, and F_1 survival. Chromosomal reciprocal translocations and somatoplastic sterility have been proposed as causes for inter-species sterility in *Melilotus*.

In addition to the inter-sterility and flower colour difference, a number of minor deviations in other morphological features have been reported, although the taxonomic value of these is still questionable. Differences in phenology, ecology, physiology/biochemistry, seed protein profiles, chromosome structure and DNA sequences provide additional evidence that the physiology and chemistry of *M. albus* and *M. officinalis* are divergent, as would be expected between genetically isolated taxa.

The definition of "species" has perplexed and eluded biologists for many years (Hey 2001), yet it is the cornerstone of biological classification systems and critical to our understanding of biodiversity. No universally applicable definition has been devised which adequately captures the diversity of life. In its application, the term "species" has become almost as variable as the biodiversity it tries to organize and perhaps nowhere has this more challenging than among domesticated crop plants. The two fundamental requirements of a species (of plant) as expressed by Gleason (1952), include "morphological distinction and genetic continuity". In more modern terms, these requirements might be expressed as the correlation of distinct states in independent characters which are uniquely maintained through reproductive isolation. Granted there are few distinctive and readily visible

morphological characteristics between *M. albus* and *M. officinalis*, but the genetic evidence reviewed here (reproductive barriers, biochemistry, DNA sequences, etc.) indicates that they are evolutionarily separated and genetically more closely related to other species in the genus than they are to each other. There is a clear correlation of distinct and independent character states which are isolated through the reproductive incompatibility of the two taxa. It is our contention that the long tradition of considering these as two different species should be maintained.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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