Eco-geographically divergent diploids, Caucasian clover (*Trifolium ambiguum*) and western clover (*T. occidentale*), retain most requirements for hybridization

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**INTRODUCTION**

A wide range of genetic mechanisms lie at the cause of sexual incompatibilities among populations, leading to speciation. Among others, these include ploidal variations, chromosomal changes and genic incompatibilities (Wolf et al., 2010). In a plants, genetic speciation mechanisms can be active anywhere from pre-pollination to endosperm or embryo failure and/or hybrid sterility (Rieseberg and Blackman, 2010).

Section *Trifolium* of the genus *Trifolium* (Ellison et al., 2006) was delineated by DNA sequence phylogeny as a clade of clover species most closely related to white clover (*T. repens* L.). This group is widely spread across Europe, western Asia and North Africa and has species with divergent adaptations that are also geographically isolated. Although natural interspecific hybridization is uncommon in *Trifolium* as a whole and in this section (Zohary and Heller, 1984), artificial hybrids have been made among several pairs of species (Williams, 1987; Abberton, 2007), suggesting that this section contains a complex of species that have radiated widely from a recent common ancestor. Further evidence for such a lineage was the finding of a unique centromeric satellite DNA repeat sequence common to several of these species (Ansari et al., 2004). This divergence has been accompanied by ploidal variation, with most species being diploid, but with at least three tetraploid species and one hexaploid. Thus ploidal variation is a likely contributor to speciation in this radiating plant group.

However, two of the most contrasting species in the section are diploid: Caucasian (or Kura) clover, *Trifolium ambiguum*, and western clover, *T. occidentale*. These species have very different morphologies, geographic locations and habitats. *Trifolium ambiguum* occurs naturally to very high altitudes (2750 m) in Turkey, Romania, Crimea, Caucasus, Transcaucasus, Iran and Iraq (Zohary and Heller, 1984). It is adapted to a range of habitats, including fields, river banks, forest fringes and steep rocky screes, and tetraploid and hexaploid forms also occur. Generally, populations adapted to the highest altitudes were diploid and those from the lowest altitudes were hexaploid with tetraploids between, but there were overlaps (Bryant, 1974). Morphologically, although distinctions among the ploidal levels were overlapping, diploid...
populations tended to have more rounded leaves, shorter florets and pistils, smaller pollen and fewer rhizomes per plant than the higher ploidies (Kannenberg and Elliott, 1962).

By contrast, *T. occidentale* is a diploid, coastal species with well-characterized populations known from the Gulf Stream coasts of Portugal, Spain, France, United Kingdom, Ireland (Coombe, 1961; Gehu, 1973; Rivas-Martinez, 1976; Preston, 1980; Kay and Ab-Shukor, 1988). *Trifolium occidentale* is a stoloniferous perennial, superficially resembling *T. repens* but with a set of characteristics that together distinguish it from *T. repens*. These characters include hairy petioles and peduncles and thick, opaque leaves with shiny under-surfaces (Coombe, 1961). *Trifolium occidentale* is very closely related to *T. repens*, and is probably one of the ancestral diploid progenitors (Ellison et al., 2006, Hand et al., 2008). *Trifolium occidentale* and tetraploid *T. repens* have the unique centromeric repeat TrR350 on all or most chromosomes, while diploid *T. ambiguum* shares the same sequence on one chromosome pair (Ansari et al., 2004).

Despite the wide geographic and ecological separations of *T. ambiguum* and *T. occidentale*, the genetic similarities between them and their other close relatives were suggestive of relatively recent and rapid speciation. Based on this hypothesis, an attempt was made to achieve interspecific hybridization between these diploids to determine whether the speciation process was genetically complete, or whether gene flow was still possible. The present paper reports, for the first time, the development of partially fertile hybrids between diploid *T. ambiguum* and *T. occidentale*, and provides descriptions of derived progeny populations and a chromosome-doubled form. Results indicated that, despite wide geographic separation and ecological and morphological differentiation, these species have partially retained most of the genetic compatibilities needed for hybrid formation. This result also has relevance for future use of wide hybridization in breeding clovers.

**MATERIALS AND METHODS**

Plant materials were obtained from the Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand. A single 2× *Trifolium ambiguum* M. Bieb. plant (AZ 564-2) ‘Summit’ was used as the female parent. Several 2× *T. occidentale* Coombe plants were used as male parents. These were from collections OCD 1158 (Faro de Finisterra), OCD 1162 (Faro de Cabo Villano), OCD 1168 (Punta Frouxeira) and OCD 1172 (Playa de San Antolin) from the coast of northern Spain.

**Hybridization and embryo rescue**

Plants were grown in a heated, insect-free glasshouse with a temperature range of 15–28 °C and a photoperiod of at least 16 h. Natural daylength was extended, when necessary, by low-intensity artificial lighting. Interspecific hybridization was carried out by hand-pollination (Williams and Verry, 1981). After 8 d, heart-stage embryos were removed from ovules under aseptic conditions and placed on a shoot-proliferating medium (CR7; White and Voisey, 1994). Four weeks later, developing shoots were transferred to a root-initiation medium (CR5; White and Voisey, 1994) for 8–12 weeks, before finally being planted into potting mix in the glasshouse. Putative hybrids were grown to maturity in the glasshouse and confirmed using the isozyme systems SDH and PGI (Williams et al., 1998) and DNA sequencing of the nuclear ITS and chloroplast *TrnL* intron regions (Ellison et al., 2006).

At maturity, the plants were clonally propagated by cuttings and flowering copies were placed outside for a full seasonal cycle to be open-pollinated (OP) by bees with the parental species and white clover. Seed-set under these conditions provided an indication of female fertility. Male fertility was estimated by extracting mature pollen, staining with 1% aceto-carmine, and counting the proportion of full-sized, fully stained grains in a minimum of 300 grains at ×200 magnification. Flowering plants were also maintained in a greenhouse and hand-pollinated, as above. Seeds from these crosses were harvested from dried heads after 4–8 weeks.

**Conventional and molecular cytogenetics**

Somatic chromosome preparations were obtained from actively growing root tips using flame-drying after enzymatic maceration as described by Ansari et al. (1999). These preparations were used both for fluorescence *in situ* hybridization (FISH) experiments and for conventional mitotic cytology after staining with Giemsa. Meiotic chromosome preparations were obtained by squashing of pollen mother cells from young floral buds either after enzymatic maceration to be used for FISH experiments or after staining with alcoholic hydrochloric acid carmine (Snow, 1963) for conventional meiotic cytology.

The DNA probes for FISH experiments were pTrS (GenBank accession AF 072692), a 596-bp fragment from *T. repens* representing part of the 5S rDNA gene family, and pTr18S (GenBank accession AF 071069), a 1.8-kb fragment from *T. repens* containing almost the entire 18S rDNA sequence. The 18S rDNA is part of the cluster of 18S-5.8S-26S rDNA genes which constitute the nucleolus organizer region (NOR). In double-colour FISH experiments, the two types of probes were individually labelled with direct fluorochrome-labelled nucleotides Cy3-dCTP or FluorX-dCTP (Amersham Pharmacia) by nick translation according to manufacturer’s specifications. Procedures for *in situ* hybridization, post-hybridization stringent washing and DAPI counterstaining of chromosomes have been described (Ansari et al., 1999). Images were captured on a cooled CCD camera (Zeiss) and were analysed using ISIS FISH imaging software (Metasystems, Germany).

Total DNA was prepared from a 2-mm² piece of leaf in 20 µL of PCR-compatible buffer using the single-step procedure of Thompson and Henry (1995). ITS amplifications were performed in 50-µL reactions using 1 µL of the prepared DNA, 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), each dNTP at 0.2 mM, each primer at 0.4 µM, and 2.5 units of Taq polymerase. ITS1, 5.8s and ITS2 were amplified as a single molecule using flanking primers EC-1 (5′-GAGGAAGGAGAAGTCGTAAC-3′) and EC-2 (5′-GGTGCGCTCGCGTTACTAAG-3′) based on the sequence reported for *Vicia faba* (Yokota et al., 1989). Cycling conditions for amplification and subsequent analysis are described by Williams et al. (2001).
Flow cytometry

Ice-cooled leaf samples were sent to Plant and Food Research, Lincoln, New Zealand, where they were analysed according to the method described by Otto (1990). Briefly, a small amount of leaf tissue was chopped finely with a razor blade in 400 μL of extraction buffer (2-0 % citric acid, 0-5 % Tween) and filtered through 30-μm mesh (Partec filter). Samples were kept on ice no longer than 3 h. Before flow cytometry, 1-6 mL of staining solution was added (2 mg mL⁻¹ DAPI in saturated dibasic sodium phosphate to give a final concentration of DAPI 2-5 μg mL⁻¹). To provide an unbiased relative measure of DNA content, all samples were co-chopped with leaf tissue of Bellis perennis as an internal standard and only readings with a coefficient of variation of <3 % were used to minimize experimental error. DAPI fluorescence was measured in a Partec PAII Flow cytometer with relative fluorescence compared with karyotyped control plants. Data were analysed using Flowmax software.

Colchicine doubling

Vegetative axillary meristems were removed from mature clonal copies of hybrid 65 in winter, when the plants were not flowering. These were cultured in Petri dishes on WCSP medium for 4 d and then transferred to WCSP + 0-1 % colchicine for 78 h, before being transferred back to WCSP. WCSP is a modification of B5 medium (Gamborg et al., 1968) and was prepared by adding 0-15 mg of 6-(γ-γ dimethylallylamino)-purine (2ip) and 0-15 mg indole-3-acetic acid (IAA) to 1 L of medium (Hussain et al., 1997). Before sub-culturing every 2 weeks over 4 months, 26 plantlets were transferred to pots containing potting mix in a greenhouse. After 10 weeks, most grew into mature plants with multiple flowering stolons. These were initially tested for chromosome doubling by pollen staining with 1 % aceticarmine (looking for a marked increase in the frequency of stained pollen grains) and observing the shape of dry pollen under a compound microscope (tetrahedral pollen indicated likely doubling, while oval pollen was diagnostic of un-doubled haploid pollen). One plant (number 20, hence referred to as hybrid 65-20) showed a small sector consisting of rhizomes from a self-pollination of T. ambiguum (65 Summit (AZ 564-2) OCD 45-13) showed a small sector consisting of rhizomes from a self-pollination of T. ambiguum

Table 1. Identification numbers and parentages of confirmed hybrids between T. ambiguum (2x) and T. occidentale (2x)

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Female parent</th>
<th>Male parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>Summit (AZ 564-2)</td>
<td>OCD 45-13</td>
</tr>
<tr>
<td>118-1</td>
<td>Summit (AZ 564-2)</td>
<td>OCD 48-17</td>
</tr>
<tr>
<td>118-2</td>
<td>Summit (AZ 564-2)</td>
<td>OCD 48-17</td>
</tr>
</tbody>
</table>

leaves. Each cutting was grown in sand/peat potting mix in 15 cm diameter plastic pots. The greenhouse was maintained at 15–25 °C with natural daylength. The pots were watered twice weekly to maintain field capacity and a complete soluble fertilizer (Yates Thrive®) was applied 2-weekly to maintain high soil fertility levels. Morphological data were recorded non-destructively in the following spring (October 2008), when the plants were flowering, and then destructively in mid-summer (January 2009). The qualitative comparisons made are listed in Table 2 and the quantitative traits recorded are listed in Table 3 (spring) and Table 4 (summer).

Analyses of variance of the quantitative traits were carried out using GENSTAT (10th edition). Data for diameter of the largest new root were square root transformed.

RESULTS

Following pollination of AZ 564-2 with several diploid T. occidentale plants, the frequency of pod development was high (approx. 80–90 %) and considerable numbers of small green heart-stage embryos were formed. Although large numbers of these were cultured, only six produced explants that were transplanted into potting mix in pots. Two of these explants died in a few weeks, but four grew strongly and produced robust mature flowering plants. Three (Table 1) were confirmed as hybrids between 2x T. ambiguum and 2x T. occidentale but the fourth, which had apparently arisen from a self-pollination of T. ambiguum, was discarded.

General description of the plants

The general characteristics of the plants are summarized in Table 2 which shows that traits were inherited from both parent species. All three plants were similar in morphology and intermediate between the parent species. The hybrids had inherited stolons from T. occidentale and rhizomes from T. ambiguum. However, close scrutiny revealed that young petioles were hairy (a T. occidentale trait), but leaves were dull on the lower surface (a T. ambiguum trait). Stems were abbreviated and densely branched (rather than elongated like T. occidentale). Inflorescences were axillary, as in T. occidentale, but, like T. ambiguum, the florets were white, turning to pink after anthesis.

Spring measurements (non-destructive)

Detailed morphological comparisons of the plants during spring are given in Table 3. The T. occidentale plant was more highly branched (vegetative growing points) and more leafy (leaves per plant) than the T. ambiguum parent. The
Table 2. Vegetative and floral comparison of diploid *T. occidentale* (OCD 54-14) (OCD), diploid *T. ambiguus* (AZ 564-2) (AMD), three diploid interspecific hybrids (118-1, 118-2, 65) and one artificial tetraploid derivative of hybrid 65 (65-20)

<table>
<thead>
<tr>
<th>Nodal roots</th>
<th>Leaf colour</th>
<th>Flower colour</th>
<th>Flower size</th>
<th>Leaf surface</th>
<th>Growth habit</th>
<th>Young petiole</th>
<th>Flowering pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCD +</td>
<td>DG</td>
<td>Cream</td>
<td>Small</td>
<td>Shiny</td>
<td>Stolons</td>
<td>Hairy</td>
<td>Axillary</td>
</tr>
<tr>
<td>AMD 0</td>
<td>LG</td>
<td>WTP</td>
<td>Large</td>
<td>Dull</td>
<td>Rhizomes</td>
<td>Smooth</td>
<td>Terminal</td>
</tr>
<tr>
<td>118-1 OCD</td>
<td>INT AMD</td>
<td>AMD AMD</td>
<td>AMD AMD</td>
<td>St + Rz</td>
<td>OCD</td>
<td>OCD</td>
<td></td>
</tr>
<tr>
<td>118-2 OCD</td>
<td>INT AMD</td>
<td>AMD AMD</td>
<td>AMD AMD</td>
<td>St + Rz</td>
<td>OCD</td>
<td>OCD</td>
<td></td>
</tr>
<tr>
<td>65 OCD</td>
<td>INT AMD</td>
<td>AMD AMD</td>
<td>AMD AMD</td>
<td>St + Rz</td>
<td>OCD</td>
<td>OCD</td>
<td></td>
</tr>
<tr>
<td>65-20 OCD</td>
<td>AMD AMD</td>
<td>AMD AMD</td>
<td>AMD AMD</td>
<td>St + Rz</td>
<td>OCD</td>
<td>OCD</td>
<td></td>
</tr>
</tbody>
</table>

The hybrids are described as being similar to *T. occidentale* (OCD), similar to *T. ambiguus* (AMD), intermediate (INT) or having characteristics of both (St + Rz). Other abbreviations: DG, dark green; LG, light green; WTP, white turning pink after anthesis.

Table 3. Mean morphological data from non-destructive observations in spring of three diploid and one tetraploid interspecific hybrid between AMD (*T. ambiguus* 2x) and OCD (*T. occidentale* 2x) in comparison with parental plants

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>VGP</th>
<th>LPP</th>
<th>PL</th>
<th>LT</th>
<th>ST</th>
<th>PS</th>
<th>FN</th>
<th>DF</th>
<th>RPP</th>
<th>ORD</th>
<th>DLNR</th>
<th>RZPP</th>
<th>RZD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCD</td>
<td>2x</td>
<td>118</td>
<td>166</td>
<td>94</td>
<td>0.22</td>
<td>2.2</td>
<td>73</td>
<td>7.5</td>
<td>89</td>
<td>12</td>
<td>2.4</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>AMD</td>
<td>2x</td>
<td>16.5</td>
<td>44</td>
<td>169</td>
<td>0.16</td>
<td>2.8</td>
<td>54</td>
<td>1.3</td>
<td>105</td>
<td>9</td>
<td>4.8</td>
<td>3.8</td>
<td>5.8</td>
</tr>
<tr>
<td>118-1</td>
<td>2x</td>
<td>43.5</td>
<td>75</td>
<td>112</td>
<td>0.15</td>
<td>2.7</td>
<td>38</td>
<td>8.8</td>
<td>85</td>
<td>26</td>
<td>2.5</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>118-2</td>
<td>2x</td>
<td>47.5</td>
<td>73</td>
<td>114</td>
<td>0.13</td>
<td>2.5</td>
<td>39</td>
<td>11.8</td>
<td>90</td>
<td>32</td>
<td>3.6</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>65</td>
<td>2x</td>
<td>38.5</td>
<td>93</td>
<td>141</td>
<td>0.14</td>
<td>2.9</td>
<td>33</td>
<td>18.5</td>
<td>86</td>
<td>15</td>
<td>2.9</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>65-20</td>
<td>4x</td>
<td>57.0</td>
<td>107</td>
<td>145</td>
<td>0.15</td>
<td>3.3</td>
<td>56</td>
<td>18.8</td>
<td>88</td>
<td>15</td>
<td>3.4</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Significance: *** = Significant differences (*P* < 0.001); ** = Significant differences (*P* < 0.01); * = Significant differences (*P* < 0.05); n.s. = no significant differences; NA = not analysed.

Below ground, the hybrids showed positive transgressive expression for numbers of roots per plant, the hybrids having more roots than either parent. *Trifolium ambiguus* roots were considerably thicker than those of *T. occidentale*, and the hybrids were intermediate in thickness, for both the original root and the thickest new roots. (The three thickest new roots were measured and all gave similar results. Data were

hybrid plants were intermediate in branching and leafiness between the parents. The hybrids were also intermediate for petiole length where the *T. ambiguus* parent was considerably larger than the *T. occidentale* plant. *Trifolium occidentale* had markedly thicker leaves than *T. ambiguus* and the hybrids were similar to or thinner than the *T. ambiguus* parent. *Trifolium occidentale* had thinner stems than *T. ambiguus* and the hybrids were generally as thick as *T. ambiguus*. *Trifolium occidentale* had thicker leaves than *Trifolium occidentale* had larger than *T. ambiguus* and the hybrids were intermediate in thickness, for both the original root and the thickest new roots. (The three thickest new roots were measured and all gave similar results. Data were
presented only for the thickest.) All of the hybrids inherited the ability to form rhizomes from the *T. ambiguum* parent. However, the numbers of rhizomes were significantly lower than this parent. Rhizomes of hybrid 65 were thinner than those of the other hybrids.

**Summer measurements (destructive harvest)**

Results of this harvest are given in Table 4. As before, the *T. occidentale* plant was markedly more leafy and highly branched than the *T. ambiguum* parent, with the hybrids in between, but tending to be closer to *T. ambiguum*. By this time, flowering of *T. occidentale* was nearly finished, and flower numbers were markedly higher for the hybrids than for either parent. Shoot dry weights, both total and above cutting height, were higher for the *T. occidentale* plant than for the hybrids and *T. ambiguum*, which, within the group, did not significantly differ. By contrast, the *T. ambiguum* plant root system was approximately five times larger than that of the *T. occidentale* plant and more than twice hybrid 65. All hybrids produced rhizomes, but these contributed only 2–3% to the dry weight of the total root-rhizome system, as opposed to 16% in *T. ambiguum*. The total below-ground dry weight of the *T. ambiguum* plant was approx. 2.5 times that of the best hybrid. Total plant dry weight (stem + root + rhizomes) was highest for *T. ambiguum*, while *T. occidentale* and the hybrids were markedly smaller.

**Somatic chromosomes**

Somatic chromosome numbers of the three hybrids were $2n = 16$ (Figs 1A, 2A and B). In hybrid 65, two NORs were present but, in many cells, one NOR tended to be condensed...
and difficult to identify. FISH analysis using 18S and 5S rDNA probes (Fig. 2) was used to distinguish the NOR chromosomes, where the T. occidentale-derived chromosome carried 5S rDNA and the T. ambiguum-derived chromosome lacked 5S rDNA (Ansari et al., 1999). A 5S rDNA-carrying chromosome from T. ambiguum was identified by a large signal, and a second 5S chromosome from T. occidentale carried a very small signal (Ansari et al., 1999).

Meiosis

Meiotic configurations (Table 5 and Fig. 2) in pollen mother cells of hybrid 65 were predominantly bivalent, with a low frequency of univalents and no multivalents. Disjunction at anaphase I was consistently 8–8.

Fertility and seed-set

Pollen stainability results for all three hybrids indicated low male fertility. Hybrid 65 showed poor pollen dehiscence and was assessed for pollen stainability on three occasions, giving 5/300, 3/400 and 25/400 for a mean of 3.0%. Hybrids 118-1 and 118-2 were similar at 2.5% and 2.0%, respectively. Female fertilities were determined from seed-sets following open pollination and hand crosses.

Seed-set following open pollination

Plants open pollinated in the presence of both parent species, along with T. repens and 4x and 6x T. ambiguum plants, were observed to develop pods. A few of these pods were harvested while green and the majority were left to develop normally. The green pods contained a few developing seeds from which the embryos were extracted and placed in tissue culture, as for the original crosses. These produced six mature progeny plants which flowered.

The pods that were left to develop normally on hybrid 65 yielded seeds at a frequency of 119/149 (0.8) seeds/head. To date, several hundred OP seeds have been harvested. Hybrids 118-1 and 118-2 were similar at 2.5% and 2.0%, respectively. Female fertilities were determined from seed-sets following open-pollination and hand crosses.

Seed-set following hand crosses

None of the hybrids produced seeds when self pollinated or when inter-crossed among themselves. The only successful hand crosses were between the hybrids as female and diploid T. occidentale as male. Hybrid 118-2 gave no seeds when pollinated with T. occidentale (seven heads), 118-1 gave two seeds (from six heads) and hybrid 65 produced 30 seeds from 19 heads. A few backcrosses (eight heads) to diploid T. occidentale (next section). The remainder had variable higher ploidal levels (FC = 4.9–6.6) and these have not been verified or further analysed. The putative triploids all had estimated pollen fertilities of 20–30% and exhibited slender stoloniferous growth. Very few seeds (approx. 1.0 per 500 florets) were produced following pollination with white clover. Several of the plants with apparent higher ploidies were robust with thick stolons and roots and pollen fertilities of 18–72% (median 37%) among the seven that

<p>| Table 5. Somatic chromosome numbers and meiotic configurations in 2x hybrid 65 and colchicine-doubled 4x hybrid 65-20 |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Plant</th>
<th>Somatic chromosome number</th>
<th>No. of PMCs</th>
<th>Meiotic configurations [mean (range)]</th>
<th>Anaphase I disjunction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid 65</td>
<td>2n = 2x = 16</td>
<td>83</td>
<td>I: 1.6 (0–4)</td>
<td>7.2 (6–8)</td>
</tr>
<tr>
<td>Hybrid 65-20</td>
<td>2n = 4x = 32</td>
<td>71</td>
<td>II: 2.1 (0–7)</td>
<td>11.5 (7–14)</td>
</tr>
</tbody>
</table>

<p>| Table 6. Characteristics of 12 random OP progeny plants from hybrid 65 |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Plant no.</th>
<th>FC, PS</th>
<th>Stem type</th>
<th>Below-ground type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.3</td>
<td>18</td>
<td>Thick stolons</td>
</tr>
<tr>
<td>2</td>
<td>6.3</td>
<td>72</td>
<td>Thick, possible rhizomes</td>
</tr>
<tr>
<td>3</td>
<td>6.4</td>
<td>49</td>
<td>Thick stolons</td>
</tr>
<tr>
<td>4</td>
<td>6.6</td>
<td>41</td>
<td>Thick stolons</td>
</tr>
<tr>
<td>5</td>
<td>5.4</td>
<td>24</td>
<td>Thick stolons</td>
</tr>
<tr>
<td>6</td>
<td>5.3</td>
<td>20</td>
<td>Stolons</td>
</tr>
<tr>
<td>7</td>
<td>5.3</td>
<td>44</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>2.9</td>
<td>30</td>
<td>Long, thin stolons</td>
</tr>
<tr>
<td>9</td>
<td>4.9</td>
<td>n.f.</td>
<td>Stolons</td>
</tr>
<tr>
<td>10</td>
<td>2.8</td>
<td>20</td>
<td>Thin stolons</td>
</tr>
<tr>
<td>11</td>
<td>6.3</td>
<td>33</td>
<td>Upright stems</td>
</tr>
<tr>
<td>12</td>
<td>2.9</td>
<td>27</td>
<td>Long, thin stolons</td>
</tr>
</tbody>
</table>

FC, Approximate ploidal level (flow cytometry); PS, pollen stainability (%); n.f., plant did not flower; n.d., not determined.
flowered. Only two of these plants (−2, −3) produced selfed seeds but six produced low numbers of seeds (1–9 seeds per 100 florets) when pollinated with white clover.

Plants from crosses with diploid T. occidentale

The progeny plants tested so far from crosses between hybrids 65 and 118-1 with 2n T. occidentale (OCD) were triploid (2n = 24; Figs 1D, 2C and D). There were three satellites (Fig. 1D), two of which were distal to extended NOR regions and one to a condensed NOR. FISH (Fig. 2D) showed the genomic constitution of one plant, hybrid (65 × OCD)-9 to be AOO (1 ambiguum: 2 occidentale), indicating that an unreduced female gamete (AO) from hybrid 65 had fused with a haploid male gamete (O) from T. occidentale. FISH also showed (Fig. 2C and D) that the two NORs from T. occidentale were decondensed and the NOR from T. ambiguum was condensed. These plants morphologically resembled hybrid 65. However, the flower heads were creamy-pink rather than white and the standard petals tended to have frilled edges. Pollen stainability results indicated higher male fertilities than hybrid 65 (Table 7). No seeds resulted following self- or cross-pollination among this triploid progeny group. However, seeds were sometimes obtained from hand-crosses between T. ambiguum and T. occidentale (Ferguson et al., 1990). The parent species are from widely separated geographic regions (eastern Europe/western Asia and western Europe) and very divergent ecological adaptations (alpine and coastal). Nevertheless, they did hybridize to produce fertile progeny. The T. occidentale plants used in the present work were from populations on the northern coast of Spain that were clearly isolated reproductively from T. ambiguum populations from the Caucasus. The successful hybridization, albeit difficult, thus represents a coming together of two reproductively isolated species that must have relatively recently diverged from a common lineage, as is also indicated by DNA sequence phylogeny (Ellison et al., 2006) and the partial sharing of a unique centromeric satellite sequence (Ansari et al., 2004).

On present knowledge, the possibility of gene flow between T. ambiguum and uncharacterized taxa in the eastern Mediterranean cannot be totally eliminated. Zohary and Heller (1984) placed T. occidentale in T. repens var. biasolettii, which, in addition to western European coastal populations (i.e. T. occidentale), included populations from Greece, the Balkans and western Turkey. Coombe (1961) argued compellingly that T. occidentale was distinctive from the more eastern T. repens var. biasolettii. Because T. repens var. biasolettii remains uncharacterized, it is currently uncertain whether it is close to diploid T. occidentale or tetraploid T. repens. Nevertheless, it is clear that western T. occidentale populations are reproductively isolated from both T. repens var. biasolettii and T. ambiguum by distance. It is also likely, but less certain, that T. ambiguum is isolated by distance from the eastern populations of T. repens var. biasolettii.

Successful crossing of these species to produce partially fertile hybrids has demonstrated that nearly all of the steps in naturally occurring hybrid formation from pollination onward are, at least partially, genetically intact. The one possible exception is endosperm development which, because embryo rescue was applied in all cases, was not tested. Pre-pollination barriers were not systematically studied, but our long-term experience and studies of the progeny of crosses has shown that pollen is freely transferred by bees between T. ambiguum and T. occidentale. Thus it appears likely that, in this case, we are observing the results of progressive accumulation of small genetic changes that have occurred in these species over a period of separate

### DISCUSSION

This is the first report of partially fertile hybrids between T. ambiguum (AA) and T. occidentale (OO). Previously, one sterile (non-flowering) hybrid had been reported between hexaploid T. ambiguum and diploid T. occidentale (Ferguson et al., 1990). The parent species are from widely separated geographic regions (eastern Europe/western Asia and western Europe) and very divergent ecological adaptations (alpine and coastal). Nevertheless, they did hybridize to produce fertile progeny. The T. occidentale plants used in the present work were from populations on the northern coast of Spain that were clearly isolated reproductively from T. ambiguum populations from the Caucasus. The successful hybridization, albeit difficult, thus represents a coming together of two reproductively isolated species that must have relatively recently diverged from a common lineage, as is also indicated by DNA sequence phylogeny (Ellison et al., 2006) and the partial sharing of a unique centromeric satellite sequence (Ansari et al., 2004).

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development. If so, then these small changes were unlikely to have been the genetic drivers of speciation. Rather, the results are consistent with speciation having occurred as the result of a larger event that led to the source populations separating and undergoing evolution in geographic and ecological isolation. Subsequently, small genetic changes have accumulated but have not yet reached the point at which they completely prevent hybridization from occurring. This is consistent with the genetic model of speciation suggested by Venditti et al. (2010) in which speciation is driven by rare stochastic events rather than by accumulation of small genetic changes. If so, then the small group of about 15 closely related, but highly divergent, *Trifolium* species in section *Trifoliumstrum* might provide a very useful group in which to study the genetics of speciation. This group includes long-lived perennials as well as annuals, ploidal variations and at least one possible case of aneuploidy (Ellison et al., 2006).

The agronomic significance of the hybrids

There has been considerable interest in *T. ambiguum* as an agricultural species for grazed pastures, especially in Australia (Bryant, 1974; Dear and Zorin, 1985; Hill and Mulcahy, 1993), New Zealand (Stewart and Daly, 1980; Black et al., 2006), and North America (Speer and Allinson, 1985; Taylor and Smith, 1998; Brummer and Moore, 2000). The agronomic traits of interest were generally persistence, drought tolerance and resistance to viruses (Barnett and Gibson, 1975). However, the species has several weaknesses, including slow establishment and poor seed production (Bryant, 1974), and very specific rhizobial-strain requirements for nodulation (Hely, 1957; Pryor et al., 1998). These weaknesses made it a marginal candidate for sown pastures. Most of the commercial varieties or germplasm releases that were developed were hexaploid, but cultivars of diploid *T. ambiguum* were released, including ‘Alpine’ (Anonymous, 1983), ‘Summit’ (Barnard, 1972) and ‘Forest’ (Mackay, 1982).

Interspecific hybridization might provide a better strategy for exploiting the advantages of *T. ambiguum*. The agronomic advantages of *T. ambiguum* are largely complementary to weaknesses in white clover (*T. repens* L.), and vice versa (Williams, 1987). Therefore hybridization between *T. ambiguum* and *T. repens* has sometimes been seen as a desirable strategy for the potential improvement of both species (Williams, 1987; Meredith et al., 1995). However, within section *Trifoliumstrum*, DNA sequence comparisons among the species suggest *T. ambiguum* is the most distantly related to white clover (Ellison et al., 2006). This distance was reflected in the relative difficulty in achieving hybrids between *T. ambiguum* and *T. repens*, and fertile hybrids are rare (Williams and Verry, 1981; Meredith et al., 1995). In addition, there is little homoeologous chromosome pairing in these hybrids, and backcrossing to white clover leads initially to hexaploid rather than tetraploid populations (Meredith et al., 1995).

In the present study, meiotic analysis showed that chromosome pairing frequently occurred between all eight homoeologues in the diploid hybrid 65 (Table 5; Fig. 1B). The formation of multivalents in the colchicine-doubled form (65-20) also indicated homoeologous pairing (Fig. 1F). Not only were these results consistent with a comparatively recent species divergence, but they also suggested that genetic recombination could occur between the genomes of the two species. This would open up some new plant breeding opportunities, including the ability to create new hybrid populations that represent artificial new species at both the diploid (AO) and amphidiploid (AAOO) levels.

When hybrid 65 (AO) was backcrossed to *T. occidentale* (OO), the progeny were triploid rather than the expected diploid. Apparently unreduced gametes from the AO hybrid combined with normal gametes from *T. occidentale* to produce AOO progeny plants (presumably with some interspecific recombination). These progeny were more fertile than the *F*1 hybrid (Table 7) and may provide a solution to the problem of recombinating *T. ambiguum* and *T. repens* genomes at the tetraploid level. This would involve using *T. occidentale* as a genetic bridge between *T. ambiguum* and *T. repens*. The basis for this genetic bridge is that *T. occidentale* and *T. ambiguum* chromosomes pair, and *T. occidentale* and *T. repens* chromosomes have also been shown to pair (Chen and Gibson, 1970).

When hybrid 65 was open-pollinated, a few progeny plants were triploid (Table 6), consistent with the situation described above, where unreduced gametes from the hybrid combined with normal *T. occidentale* male gametes. In addition, several progeny plants were indicated by flow cytometry to have higher ploidal levels (5x–6x; Table 6). The derivation of such progeny from a diploid mother plant was unexpected and also suggestive of unreduced gametes. However, these plants require further analysis to elucidate their origins. Because some of them produced progeny when crossed with *T. repens*, they may be useful for clover breeding.

The phenotypes of the diploid hybrid plants sometimes showed combinations of traits from both parent species and sometimes were transgressive, falling outside the range of either parent, especially for flower numbers (Tables 3 and 4). In general, the hybrids were densely stoloniferous plants with thick roots and a few rhizomes. As such, they were attractive in plant habit, combining spreading mechanisms both above and below ground, with robust roots. The best hybrid (65) had above-ground vigour in pots similar to the poorer parent (*T. ambiguum*) and the below-ground vigour, although better than *T. occidentale*, was markedly inferior to the better *T. ambiguum* parent. Therefore, these initial *F*1 hybrids will need considerable breeding and selection, probably involving new and different genotype combinations, to generate agriculturally useful material. However, as they are also only partially fertile, the diploid hybrids are unlikely to be used in their own right, and they should be regarded as intermediates in applying the breeding approaches described above. The chromosome-doubled form (65-20), because of its higher fertility, is more likely to lead to agriculturally useful populations. However, as it was little different in vigour from its diploid progenitor, more vigorous new genotypes would be needed if the breeding of such forms is to progress.

Conclusions

*Trifolium ambiguum* and *T. occidentale* have, despite widespread geographic separation, different ecological adaptations and markedly divergent morphologies, retained virtually all of the genetic mechanisms needed for hybridization. Species
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LITERATURE CITED


