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

Warren M. Williams^{1,2*}, Isabelle M. Verry¹, Helal A. Ansari¹, S. Wajid Hussain¹, Ihsan Ullah^{1,2},
Michelle L. Williamson¹ and Nicholas W. Ellison¹

¹AgResearch Grasslands Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand and ²College of Sciences, Massey University, Palmerston North 4442, New Zealand

*For correspondence. E-mail warren.williams@agresearch.co.nz

Received: 15 April 2011 Returned for revision: 6 July 2011 Accepted: 12 July 2011 Published electronically: 31 August 2011

• **Background and Aims** DNA sequence similarities and hybridization patterns in *Trifolium* (clovers) section *Trifoliastrum* suggest that rapid radiation from a common ancestral source led to this complex of diverse species distributed across Europe, western Asia and North Africa. Two of the most geographically and ecologically divergent of these species are the rhizomatous *T. ambiguum* from high altitudes in eastern Europe and western Asia and the stoloniferous *T. occidentale* from sea level in western Europe. Attempts were made to hybridize these species to ascertain whether, despite this separation, gene flow could be achieved, indicating the retention of the genetic factors necessary for hybridization.

• **Methods** Three F_1 hybrids formed after embryo rescue were described, characterized by conventional and molecular cytogenetics, subjected to fertility tests and progeny generations were developed.

• **Results and Conclusions** Partially fertile hybrids between *Trifolium ambiguum* and *T. occidentale* were obtained for the first time. The F_1 hybrids produced seeds after open-pollination, and also produced triploid progeny in backcrosses to *T. occidentale* from the functioning of unreduced gametes in the hybrids. These plants were fertile and produced progeny with *T. occidentale* and with *T. repens*. Meiotic chromosome pairing in the F_1 showed six to eight bivalents per pollen mother cell, indicating pairing between the parental genomes. A chromosome-doubled form of one hybrid, produced using colchicine, showed some multivalents, indicative of inter-specific chromosome pairing. The hybrid plants were robust and combined phenotypic characteristics of both species, having stolons, thick roots and a few rhizomes. Results show that despite separation by the entire breadth of Europe, the speciation process is incomplete, and these taxa have partially retained most of the genetic compatibilities needed for hybridization (possibly except for endosperm development, which was not tested). The fertile progeny populations could lead to new clover breeding strategies based on new hybrid forms.

Key words: *Trifolium ambiguum*, *T. occidentale*, *T. repens*, interspecific hybridization, Caucasian clover, western clover, white clover, introgression, genetic bridge, unreduced gametes, speciation.

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 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 *Trifoliastrum* 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 2x *Trifolium ambiguum* M. Bieb. plant (AZ 564-2) 'Summit' was used as the female parent. Several 2x *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 % acetocarmine, and counting the proportion of full-sized, fully stained grains in a minimum of 300 grains at $\times 200$ 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 pTr5S (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 μ L of PCR-compatible buffer using the single-step procedure of Thompson and Henry (1995). ITS amplifications were performed in 50- μ L reactions using 1 μ L of the prepared DNA, 1 $\times$ 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 μ M, 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'-GTTCGCTCGCCGTTACTAAG-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 μ L of extraction buffer (2.0 % citric acid, 0.5 % Tween) and filtered through 30- μ m 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 μ g 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). After 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 % acetocarmine (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 approximately two stolons with a high frequency of stained pollen and tetrahedral dry pollen grains. These stolons were anchored down with staples to the soil surface to ensure that nodal roots formed, after which they were detached and planted in separate pots. Chromosome counts on root tips and meiotic preparations of pollen mother cells later confirmed that chromosome doubling had occurred.

Phenotypic description of the hybrids: pot experiment

The three 2x interspecific hybrids (Table 1) were characterized, along with the colchicine-doubled 4x hybrid (65-20), 2x 'Summit' *T. ambiguum* (AZ 564-2) and 2x *T. occidentale* (OCD 54-14) in a pot experiment in the greenhouse. The randomized complete block experiment with four replications was planted on 17 July 2008 at the Grasslands Research Centre, Palmerston North, using cuttings from mature plants. Each cutting consisted of 3–4 cm of stem with one main root, one active growing point and three or four fully open trifoliolate

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

Hybrid	Female parent	Male parent
65	Summit (AZ 564-2)	OCD 45-13
118-1	Summit (AZ 564-2)	OCD 48-17
118-2	Summit (AZ 564-2)	OCD 48-17

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. ambiguum (AZ 564-2) (AMD), three diploid interspecific hybrids (118-1, 118-2, 65) and one artificial tetraploid derivative of hybrid 65 (65-20)*

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

The hybrids are described as being similar to *T. occidentale* (OCD), similar to *T. ambiguum* (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. ambiguum 2x) and OCD (T. occidentale 2x) in comparison with parental plants*

	Ploidy	VGP	LPP	PL	LT	ST	PS	FN	DF	RPP	ORD	DLNR	RZPP	RZD
OCD	2x	118.0	166	94	0.22	2.2	73	7.5	89	12	2.4	1.0	0.0	—
AMD	2x	16.5	44	169	0.16	2.8	54	1.3	105	9	4.8	3.8	5.8	1.7
118-1	2x	43.5	78	112	0.15	2.7	38	8.8	85	26	2.5	1.9	2.0	1.5
118-2	2x	47.5	73	114	0.13	2.5	39	11.8	90	32	3.6	1.6	2.3	1.4
65	2x	38.5	93	141	0.14	2.9	33	18.5	86	15	2.9	1.9	2.0	1.1
65-20	4x	57.0	107	145	0.15	3.3	56	18.8	88	15	3.4	2.3	2.3	1.6
Significance		***	***	***	***	***	***	***	***	***	***	***	***	***
l.s.d. (0.05)		10.13	26.0	22.8	0.023	0.41	22.0	6.1	5.2	6.6	0.94	0.05	2.5	0.26
CV (%)		18.2	26.4	10.0	10.1	10.1	26.8	32.7	4.0	26.6	18.5	10.9	59.7	12.7
s.e.		3.02	18.3	16.0	0.016	0.29	15.4	3.0	3.6	4.6	0.66	0.03	1.75	0.18

VGP, Vegetative growing points per plant; LPP, leaves per plant; PL, petiole length (mm); ST, stem thickness (mm); PS, plant spread (cm); FN, flower number per plant; DF, days to flowering; RPP, number of roots per plant; ORD, diameter of oldest root; DLNR, diameter of the largest new root; RZPP, rhizomes per plant; RZD, rhizome diameter (mm).

***, Significant differences ($P < 0.001$).

TABLE 4. *Mean data from destructive harvest in summer of three diploid and one tetraploid interspecific hybrid between AMD (T. ambiguum 2x) and OCD (T. occidentale 2x) in comparison with parental plants*

	Ploidy	VGP	LPP	FN	DWGP	DWR	DWTS	DWRS	DWRZ	DWBG	TPDW
OCD	2x	276	1112	2	29.6	5.3	34.9	4.3	0.0	4.3	39.2
AMD	2x	38	192	11	18.2	5.2	23.4	26.4	5.2	31.6	55.0
118-1	2x	85	502	65	14.2	5.5	19.7	7.3	0.3	7.6	27.3
118-2	2x	98	608	98	13.9	5.8	19.7	8.0	0.2	8.2	27.9
65	2x	71	536	91	20.4	5.3	25.8	11.5	0.4	11.9	37.7
65-20	4x	68	364	119	20.2	5.3	25.5	7.4	1.3	8.7	34.2
Significance		**	**	**	**	n.s.	NA	**	**	NA	NA
l.s.d. (0.05)		48.0	135.2	35.3	7.45	2.13		7.39	3.23		

VGP, Vegetative growing points per plant; LPP, leaves per plant; FN, flowers per plant; DWGP, dry weight of leaves, inflorescences and stolon tips (simulated grazable part, g); DWR, dry weight of residual stems (after cutting); DWTS, dry weight of total shoot (g); DWRS, dry weight of root system (g); DWRZ, dry weight of rhizomes (g); DWBG – dry weight below ground; TPDW, total plant dry weight (g).

**, Significant differences ($P < 0.01$); n.s., no significant differences; NA, not analysed.

hybrid plants were intermediate in branching and leafiness between the parents. The hybrids were also intermediate for petiole length where the *T. ambiguum* parent was considerably larger than the *T. occidentale* plant. *Trifolium occidentale* had markedly thicker leaves than *T. ambiguum* and the hybrids were similar to or thinner than the *T. ambiguum* parent. *Trifolium occidentale* had thinner stems than *T. ambiguum* and the hybrids were generally as thick as *T. ambiguum*. However, for plant spread, the hybrids were smaller than the poorer parent, *T. ambiguum*. *Trifolium occidentale* and the

hybrids were earlier flowering and produced more flowers than *T. ambiguum* (Table 3). One hybrid (65) flowered more profusely than both parents.

Below ground, the hybrids showed positive transgressive expression for numbers of roots per plant, the hybrids having more roots than either parent. *Trifolium ambiguum* 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

FIG. 1. Somatic and meiotic chromosome preparations: (A) *T. ambiguum* × *T. occidentale* hybrid 65 root tip cell ($2n = 2x = 16$); (B) *T. ambiguum* × *T. occidentale* hybrid 65 metaphase I (eight bivalents); (C) *T. ambiguum* × *T. occidentale* hybrid 65 metaphase I (five bivalents, six univalents); (D) (65 × OCD)-9 ($2n = 3x = 24$) root tip cell with two decondensed NORs (dotted lines) and one condensed NOR (arrow); (E) colchicine-doubled hybrid 65-20 ($2n = 4x = 32$) root tip cell with two decondensed NORs (dotted lines) and two condensed NORs (arrows); (F) colchicine-doubled hybrid 65-20 meiotic metaphase I, showing univalent to quadrivalent configurations. Scale bars = 10 μm .

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

FIG. 2. Somatic chromosome preparations of *T. ambiguum* × *T. occidentale* hybrids. (A) DAPI-stained highly condensed late metaphase cell of hybrid 65 in grey scale, (B) the same cell counterstained with DAPI and FISH with 5S rDNA (red) and 18S rDNA (green). Arrows indicate *T. ambiguum*-derived chromosomes with either 5S rDNA or 18S rDNA signals. Arrowheads indicate *T. occidentale*-derived chromosomes with either co-localization of 5S rDNA and 18S rDNA on one chromosome, or a minor 5S rDNA signal on another chromosome. (C) DAPI-stained mid-metaphase cell of backcross hybrid (65 × OCD)-9 in grey scale. Decondensed NORs are shown by dotted lines. (D) The same cell counterstained with DAPI and FISH with 5S rDNA (red) and 18S rDNA (green). Arrows indicate *T. ambiguum*-derived chromosomes with either 5S rDNA or 18S rDNA signals. The *T. ambiguum*-derived 18S rDNA signal is highly condensed. The two decondensed NORs (green) are associated with 5S rDNA signals on the same chromosomes, indicating that these are *T. occidentale*-derived. Arrowheads indicate the other *T. occidentale*-derived chromosomes, each carrying a minor 5S rDNA signal. Scale bar = 10 μm .

TABLE 5. *Somatic chromosome numbers and meiotic configurations in 2x hybrid 65 and colchicine-doubled 4x hybrid 65-20*

Plant	Somatic chromosome number	No. of PMCs	Meiotic configurations [mean (range)]				Anaphase I disjunction
			I	II	III	IV	
Hybrid 65	$2n = 2x = 16$	83	1.6 (0–4)	7.2 (6–8)	0 (–)	0 (–)	8–8 (>40 cells)
Hybrid 65-20	$2n = 4x = 32$	71	2.1 (0–7)	11.5 (7–14)	0.6 (0–2)	1.3 (0–4)	16–16 (>20 cells) 15–17 (2 cells)

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 less studied and have each produced OP seeds.

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

TABLE 6. *Characteristics of 12 random OP progeny plants from hybrid 65*

Plant no.	FC	PS	Stem type	Below-ground type
1	5.3	18		
2	6.3	72	Thick stolons	Thick, possible rhizomes
3	6.4	49	Thick stolons	n.d.
4	6.6	41	Thick stolons	Thick roots
5	5.4	24	Thick stolons	n.d.
6	5.3	20	Stolons	n.d.
7	5.3	44	n.d.	n.d.
8	2.9	30	Long, thin stolons	Slender roots
9	4.9	n.f.	Stolons	n.d.
10	2.8	20	Thin stolons	n.d.
11	6.3	33	Upright stems	n.d.
12	2.9	27	Long, thin stolons	n.d.

FC, Approximate ploidal level (flow cytometry); PS, pollen stainability (%); n.f., plant did not flower; n.d., not determined.

and tetraploid *T. ambiguum* were unsuccessful. Crosses between hybrid 65 and one white clover plant produced a few empty testas but no full seeds.

Plants derived from embryos rescued after open-pollination

Two of the six progeny from embryo rescue were relatively highly fertile, one having pollen stainability of 48 %, and both producing hundreds of seeds when open-pollinated. Three produced no seeds, and the last plant (with pollen stainability of 28 %) gave a few seeds which produced very weak plants with variegated leaves (green/yellow or green/white).

Progeny from seeds set naturally after open-pollination

OP progeny of hybrid 65 were extremely variable in phenotype, ranging from small compact plants through slender stoloniferous plants to robust plants with very thick stolons and roots and some apparent rhizomes. One group of 12 OP progeny plants is summarized in Table 6. Based on flow cytometry analyses of leaf tissue, three of these were probable triploids (FC = 2.8–2.9), consistent with pollination by 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

TABLE 7. Pollen fertility and seed-set of triploid first backcross (BC_1) hybrids of hybrid 65 (*T. ambiguum* × *T. occidentale*) × *OCD* (*T. occidentale*)

BC_1 plant number	Pollen stain stained/total (%)	Seed-set (seeds/heads)
(65 × <i>OCD</i>)-2	180/300 (60), 159/300 (53)	× <i>OCD</i> 6/3, 0/3 × <i>OCD</i> 0/2, 0/2 × <i>AMD</i> 0/2 × <i>AMT</i> 0/2 × <i>RET</i> 0/2
(65 × <i>OCD</i>)-4	53/300 (18)	× <i>OCD</i> 1 + 1 × <i>RET</i> 0/3
(65 × <i>OCD</i>)-5	28/300 (9) 23/300 (8)	
(65 × <i>OCD</i>)-6	92/300 (31) 104/300 (35)	× <i>OCD</i> 1/2 × <i>AMT</i> 0/2
(65 × <i>OCD</i>)-7	72/300 (24)	× <i>OCD</i> 1/3 × <i>RET</i> 1/3
(65 × <i>OCD</i>)-9	81/300 (27) 90/300 (30)	× <i>OCD</i> (9/3)

RET, *T. repens*, AMD, 2x *T. ambiguum*, AMT, 4x *T. ambiguum*.

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 as female with *T. occidentale* (0–3 seeds per head). A few reciprocal crosses, as male, produced some pod and seed development but these remain unverified. One seed was obtained from pollination with *T. repens* (Table 7) and the resulting plant was verified using leaf marks.

Chromosome doubled hybrid 65-20

The somatic chromosome number of the colchicine-doubled hybrid 65-20 was confirmed as $2n = 4x = 32$ (Fig. 1E). The plant was very similar in phenotype to the F_1 hybrid 65 but with more spread and thicker stems (Table 3). Pollen stainability increased markedly from <10 % in hybrid 65 to 50–60 % in

65-20. Meiotic chromosome pairing in 65-20 had predominantly bivalent pairing along with low but regular incidences of univalents and multivalents (Table 5 and Fig. 1F). Chromosome disjunction at anaphase I was predominantly 16–16 but some irregular disjunction was observed (Table 5). No seeds formed when 65-20 was self pollinated, but it was female fertile and set seeds with various pollen sources. Confirmed hybrids were obtained by embryo rescue following crosses of 65-20 as female and genetically marked white clover. Pollination of white clover with 65-20 has produced one confirmed hybrid without the need for embryo rescue.

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).

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

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 *Trifoliastrum* 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 *Trifoliastrum*, 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 recombining *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 agronomically 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 agronomically 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

differentiation has therefore probably been initiated by physical separation than by genetic incompatibilities. Fertile hybrids have been formed using embryo rescue. These new transgressive interspecific hybrids have potential to be used as intermediates to achieve the highly desired breeding objective of introgressing *T. ambiguum* traits into white clover. Additionally, as they also have phenotypic similarities to robust white clover, when fertility is restored by chromosome doubling, they could also lead to the breeding of a new amphidiploid species for use in pastures world-wide.

ACKNOWLEDGEMENTS

We thank Dr Ross Bicknell and Sylvia Erasmuson, Plant & Food Research Ltd, Lincoln for flow cytometric analyses and Dr Dongwen Luo for statistical analyses. Technical support was provided by Helen van den Ende, John Ford and Brian McKenzie. Bruce Veit and Brent Barrett kindly reviewed the manuscript. The work was supported by AgResearch Limited (9957/2, 9957/3) and the New Zealand Foundation for Research, Science and Technology (C10X0711).

LITERATURE CITED

- Abbott MT. 2007. Interspecific hybridization in the genus *Trifolium*. *Plant Breeding* 126: 337–342.
- Anonymous. 1983. *Trifolium ambiguum* Bieb. (Caucasian clover) cv. Alpine (Reg. No. B-1g-5). Herbage Plant Register Supplement. *Journal of the Australian Institute of Agricultural Science* 49: 242–243.
- Ansari HA, Ellison NW, Reader SM, et al. 1999. Molecular cytogenetic organisation of 5S and 18S-26S rDNA loci in white clover (*Trifolium repens* L.) and related species. *Annals of Botany* 83: 199–206.
- Ansari HA, Ellison NW, Griffiths AG, Williams WM. 2004. A lineage-specific centromeric satellite sequence in the genus *Trifolium*. *Chromosome Research* 12: 1–11.
- Barnard C. 1972. *Register of Australian herbage plant cultivars*. Canberra, Australia: Division of Plant Industry, CSIRO, 148–149.
- Barnett OW, Gibson PB. 1975. Identification and prevalence of white clover viruses and resistance of *Trifolium repens* to these viruses. *Crop Science* 15: 32–37.
- Black AD, Moot DJ, Lucas RJ. 2006. Development and growth characteristics of Caucasian and white clover seedlings, compared with perennial ryegrass. *Grass and Forage Science* 61: 442–453.
- Brummer EC, Moore KJ. 2000. Persistence of perennial cool-season grass and legume cultivars under continuous grazing by beef cattle. *Agronomy Journal* 92: 466–471.
- Bryant WG. 1974. Caucasian clover (*Trifolium ambiguum* Bieb.): a review. *Journal of the Australian Institute of Agricultural Science* 40: 11–19.
- Chen CC, Gibson PB. 1970. Meiosis in two species of *Trifolium* and their hybrids. *Crop Science* 10: 188–189.
- Coombe DE. 1961. *Trifolium occidentale*, a new species related to *T. repens* L. *Watsonia* 5: 68–87.
- Dear BS, Zorin M. 1985. Persistence and productivity of *Trifolium ambiguum* M. Bieb. (Caucasian clover) in a high altitude region of south-eastern Australia. *Australian Journal of Experimental Agriculture* 25: 124–132.
- Ellison NW, Liston A, Steiner JJ, Williams WM, Taylor NL. 2006. Molecular phylogenetics of the clover genus (*Trifolium* – Leguminosae). *Molecular Phylogenetics and Evolution* 39: 688–705.
- Ferguson NH, Rupert EA, Evans PT. 1990. Interspecific *Trifolium* hybrids produced by embryo and ovule culture. *Crop Science* 30: 1145–1149.
- Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151–158.
- Gehu J-M. 1973. *Trifolium occidentale* D.E. Coombe espece nouvelle pour le littoral du nord du Portugal. *Agronomia Lusitana* 34: 197–204.
- Hand ML, Ponting RC, Drayton MC, et al. 2008. Identification of homologous, homoeologous and paralogous sequence variants in an outbreeding allopolyploid species based on comparison with progenitor taxa. *Molecular Genetics and Genomics* 280: 293–304.
- Hely FW. 1957. Symbiotic variation in *Trifolium ambiguum* M. Bieb. with special reference to the nature of resistance. *Australian Journal of Biological Science* 10: 1–16.
- Hill MJ, Mulcahy C. 1993. Seedling vigour and rhizome development in *Trifolium ambiguum* M. Bieb. (Caucasian clover) as affected by density of companion grasses, fertility, drought and defoliation in the first year. *Australian Journal of Agricultural Research* 46: 807–819.
- Hussain SW, Williams WM, Mercer CF, White DWR. 1997. Transfer of clover cyst nematode resistance from *Trifolium nigrescens* Viv. to *T. repens* L. by interspecific hybridisation. *Theoretical and Applied Genetics* 95: 1274–1281.
- Kannenberg LW, Elliott FC. 1962. Ploidy in *Trifolium ambiguum*, M. Bieb. in relation to some morphological and physiological characters. *Crop Science* 2: 378–381.
- Kay QON, Ab-Shukor NA. 1988. *Trifolium occidentale* D.E. Coombe, new to Wales. *Watsonia* 17: 168–171.
- Mackay JHE. 1982. *Trifolium ambiguum* Bieb. (Caucasian clover) cv. Forest, Reg. No. B-1g-4. *Register of Australian Herbage Plant Cultivars. Supplement to the 1972 Edition*. Canberra, Australia: Division of Plant Industry, CSIRO, 49–51.
- Meredith MR, Michaelson-Yeates TPT, Ougham HJ, Thomas H. 1995. *Trifolium ambiguum* as a source of variation in the breeding of white clover. *Euphytica* 82: 185–191.
- Otto F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *Methods in Cell Biology* 33: 105–110.
- Preston CD. 1980. *Trifolium occidentale* D.E. Coombe, new to Ireland. *Irish Nature Journal* 20: 37–40.
- Pryor HN, Lowther WL, McIntyre HJ, Ronson CW. 1998. An inoculant *Rhizobium* strain for improved establishment of hexaploid Caucasian clover (*Trifolium ambiguum*). *New Zealand Journal of Agricultural Research* 41: 179–189.
- Rieseberg LH, Blackman BK. 2010. Speciation genes in plants. *Annals of Botany* 106: 439–455.
- Rivas-Martinez S. 1976. De plantis Hispaniae notulae systematicae, chorologicae et ecologicae. 1. *Candollea* 31: 111–117.
- Snow R. 1963. Alcoholic hydrochloric acid-carmines as a stain for chromosomes in squash preparations. *Stain Technology* 38: 9–13.
- Speer GS, Allinson DW. 1985. Kura clover (*Trifolium ambiguum*): legume for forage and soil conservation. *Economic Botany* 39: 165–176.
- Stewart AV, Daly GT. 1980. Growth of an established stand of *Trifolium ambiguum* in a fertile lowland environment. *New Zealand Journal of Experimental Agriculture* 8: 255–257.
- Taylor NL, Smith RR. 1998. Kura clover (*Trifolium ambiguum* M.B.) breeding, culture, and utilization. *Advances in Agronomy* 63: 153–178.
- Thompson D, Henry R. 1995. Single step protocol for preparation of plant tissue for analysis by PCR. *BioTechniques* 19: 394–400.
- Venditti C, Meade A, Pagel M. 2010. Phylogenies reveal new interpretation of speciation and the Red Queen. *Nature* 463: 349–352.
- White DWR, Voisey C. 1994. Prolific direct regeneration from cotyledons of white clover. *Plant Cell Reports* 13: 303–308.
- Williams EG, Verry IM. 1981. A partially fertile hybrid between *Trifolium repens* and *T. ambiguum*. *New Zealand Journal of Botany* 19: 1–7.
- Williams WM. 1987. Genetics and breeding. In: *White clover*. Baker MJ, Williams WM. eds. Wallingford, UK: CAB International, 343–419.
- Williams WM, Mason KM, Williamson ML. 1998. Genetic analysis of shikimate dehydrogenase allozymes in *Trifolium repens* L. *Theoretical and Applied Genetics* 96: 859–868.
- Williams WM, Ansari HA, Ellison NW, Hussain SW. 2001. Evidence of three subspecies in *Trifolium nigrescens* Viv. *Annals of Botany* 87: 683–691.
- Wolf JB, Lindell J, Backstrom N. 2010. Speciation genetics: current status and evolving approaches. *Philosophical Transactions of the Royal Society B* 365: 1717–1733.
- Yokota Y, Kawata T, Iida Y, Kato A, Tanifuji S. 1989. Nucleotide sequences of the 5-8S rDNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. *Journal of Molecular Evolution* 29: 294–301.
- Zohary M, Heller D. 1984. *The genus Trifolium*. Jerusalem: Israel Academy of Sciences and Humanities.