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Co-infection patterns and geographic distribution of a complex pathosystem targeted by pathogen-resistant plants

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Abstract. Increasingly, pathogen-resistant (PR) plants are being developed to reduce the agricultural impacts of disease. However PR plants also have the potential to result in increased invasiveness of nontarget host populations and so pose a potential threat to nontarget ecosystems. In this paper we use a new framework to investigate geographical variation in the potential risk associated with unintended release of genetically modified alfalfa mosaic virus (AMV)-resistant *Trifolium repens* (white clover) into nontarget host populations containing AMV, clover yellow vein virus (ClYVV), and white clover mosaic virus (WClMV) in southeastern Australia. Surveys of 213 sites in 37 habitat types over a 300 000-km^2 study region showed that *T. repens* is a significant weed of many high-conservation-value habitats in southeastern Australia and that AMV, ClYVV, and WClMV occur in 15–97% of nontarget host populations. However, *T. repens* abundance varied with site disturbance, habitat conservation value, and proximity to cropping, and all viral pathogens had distinct geographic distributions and infection patterns. Virus species frequently co-infected host plants and displayed nonindependent distributions within host populations, although co-infection patterns varied across the study region. Our results clearly illustrate the complexity of conducting environmental risk assessments that involve geographically widespread, invasive pasture species and demonstrate the general need for targeted, habitat- and pathosystem-specific studies prior to the process of tiered risk assessment.

Key words: alfalfa mosaic virus; clover yellow vein virus; disease resistance; environmental risk assessment; genetically modified; invasion; nontarget ecosystem; Trifolium repens; *white clover mosaic virus.*

Introduction

Plant breeders and researchers are increasingly utilizing targeted breeding or biotechnology to produce pathogen-resistant (PR) plants (Jauhar 2006, Gu et al. 2008) that have the potential to improve the efficiency and productivity of agricultural systems. However, some PR plants pose a potential threat to nontarget ecosystems that lie beyond the scope of the intended commercial release, since disease-resistant genotypes may exhibit increased weediness or invasiveness of host populations following relief from pathogen pressure, a process known as enemy release (Keane and Crawley 2002). Indeed, it has recently been shown that increased population growth rates and niche expansion of nontarget host populations could occur following introgression of disease resistance genes from genetically modified (GM) virus-resistant plants (Godfree et al. 2007, 2009*a*, *b*), with similar concerns being raised for other targeted pathosystems.

However, evaluating the risks that disease-resistant plants pose to nontarget ecosystems remains a daunting challenge (Dale et al. 2002). Apart from the fact that our knowledge of the plant traits that contribute to weediness in novel habitats is limited, even after decades of observation on the movement of plants to new environments (Browne et al. 2007, Hulme 2009), the specific role that diseases play in limiting the spatial distribution and abundance of plant hosts is usually unknown, apart from a few well-documented cases involving catastrophic diseases such as *Cryophonectria parasitica* (Paillet 2002) and *Phytophthora cinnamomi* (Shearer et al. 2008). This is especially true of entire groups of diseases, such as viruses, which have been poorly studied in non-agricultural systems (Roossinck 2010). The available evidence, however, suggests that the impacts of disease on wild host populations are likely to be subtle, and interact with factors such as habitat type (Godfree et al. 2009*b*), host density (Ferrandino 2005), host–pathogen coevolutionary dynamics (Fargette et al. 2006, Jones 2006), and the heritability of resistance traits (Conner et al. 2003).

The development of PR plants that target multi-disease pathosystems by methods such as marker assisted breeding (e.g., bean cultivars resistant to anthracnose, angular leaf spot, and rust [Ragagnin et al. 2009]), multiple pathogen-derived transgenes (e.g., squash resistant to cucumber mosaic virus [CMV], watermelon mosaic virus [WMV], zucchini yellow

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mosaic virus [ZYMV], and papaya ring spot virus [PRSV; Silora et al. 2006]), or RNA silencing (e.g., *Nicotiana benthamiana* resistance to four tospovirus species [Bucher et al. 2006]), pose an additional challenge to ecologists engaged in risk assessment. While it is known that multi-species pathosystem complexes are common in nature (Raybould et al. 2002, Xu et al. 2008), and that spatial and temporal variation in disease incidence across species can be large (García-Arenal et al. 2001), the interactive effects of different diseases in wild host-plant populations have rarely been quantified. However, it is clear that the demographic consequences of multiple disease infections within host species may differ from direct pairwise host–pathogen interactions (Murphy and Kyle 1995, Kim et al. 2010), and compensatory, synergistic, or antagonistic interactions among different pathogens generate a diverse array of possible interactions between different diseases and their hosts (Xi et al. 2007, Latham and Wilson 2008, Alves-Júnior et al. 2009).

Risk assessments involving complex pathosystems are also hindered by the fact that certain pathogens, perhaps most notably viruses, are often difficult to identify in the field (Lopez et al. 2008), and can cause asymptomatic infection of wild host plants (Roossinck 2010). Additionally, the scale of field surveys and experiments needed to untangle disease × host × environment interactions is often large, and so the few detailed studies to date that have investigated the risk of ecological release in pathosystems targeted by PR plants have usually focused on single host–pathogen systems (e.g., Godfree et al. 2009*a*, *b*). Species that are both important disease hosts and have a track record of invasiveness (e.g., many pasture plants [Lonsdale 1994]) present an additional challenge since their release from suppression by disease could potentially impact on a wide array of nontarget ecosystems. Some research underpinning risk assessment of pasture species has been undertaken (Cunlife et al. 2004, Wang et al. 2004, Kang et al. 2009), but very few ecological risk assessments have been completed for transgenic pasture species (Sandhu et al. 2008, 2009, Bagavathiannana and Van Ackerb 2010) and none to date that we are aware of for a GM pasture species resistant to multiple pathogens.

In this paper, we report the first stages in the ecological risk assessment of transgenic *Trifolium repens* (white clover) genotypes that express resistance to a range of viral pathogens that occur in agricultural systems and nontarget host populations (Spangenberg et al. 2001). *T. repens* is a well-studied model GM pasture species (Godfree et al. 2006, 2007, 2009*a*), which meets several criteria indicative of potential risk to nontarget plant communities. In Australia, *T. repens* is known to become naturalized or invasive in native plant communities (Godfree et al. 2004*b*) and is often infected by a range of viral diseases in agricultural landscapes (see (McLean 1983, McKirdy and Jones 1995, 1997, Norton and Johnstone 1998, Coutts and Jones 2002). Consequently, it has been argued that niche expansion following release from one such pathogen (clover yellow vein virus [Godfree et al. 2009*a*]) could occur if resistance genes sourced from newly developed PR genotypes were to enter nontarget populations, thus posing a threat to high conservation value native plant communities. These results are especially significant because *T. repens* is the most important pasture legume in many temperate regions of the world and is currently one of Australia's most widely grown pasture crops (Bouton et al. 2005).

Here, we develop and apply a simple new framework for assessing the risk posed by PR species that could potentially maintain large, geographically extensive populations in nontarget habitats (Fig. 1). In this framework, the early stages of risk assessment involve habitat identification, where potential habitats for further detailed study are identified, followed by field surveys, where information on the distribution and abundance of pathosystem components is collected. These data are then used to inform the development of the next stages of the tiered experimental risk assessment (see Fig. 1 for discussion of tiered risk assessment procedures, Wilkinson and Tepfer [2009] and Godfree et al. [2009*a*, *b*] for application). A significant element of this new framework involves critical decision-making early in the risk assessment process, where herbarium records, vegetation data, and species distribution models (Hill 1996) are used to identify potential host habitats, followed by determination of high-priority habitats based on government policy, regulatory concerns and conservation priorities. Obviously, as the complexity of the pathosystem increases, and the geographic distribution of the host species increases, the field survey component of the work becomes large.

The key aim of this study was to investigate spatial variation in the composition and structure of a model multi-species pathosystem and to derive lessons for the development of risk assessment protocols targeting disease-resistant host plants in general. We focused on *T. repens* populations infected with the viral pathogens alfalfa mosaic virus (AMV), white clover mosaic virus (WClMV), and clover yellow vein virus (ClYVV) over a 300 000-km^2 region of southeastern (SE) Australia within the climatic envelope predicted by Hill (1996) for *T. repens* (Stages 1 and 2 in Fig. 1). In this large region numerous nontarget plant community types could be placed at risk by the release of virus-resistant *T. repens*. We focus predominantly on the risk assessment of AMV-resistant *T. repens*, which is the most immediate GM virus-resistant pasture plant being assessed for commercial release in Australia (Office of the Gene Technology Regulator 2009*a*, *b*, *c*), and numerous studies have demonstrated that AMV infection can negatively impact the growth of cultivated *T. repens* by up to 33% (Houston and Oswald 1953, Miller 1962, Gibson et al. 1981, 1982). In addition, we also consider the structure and distribution of ClYVV– and

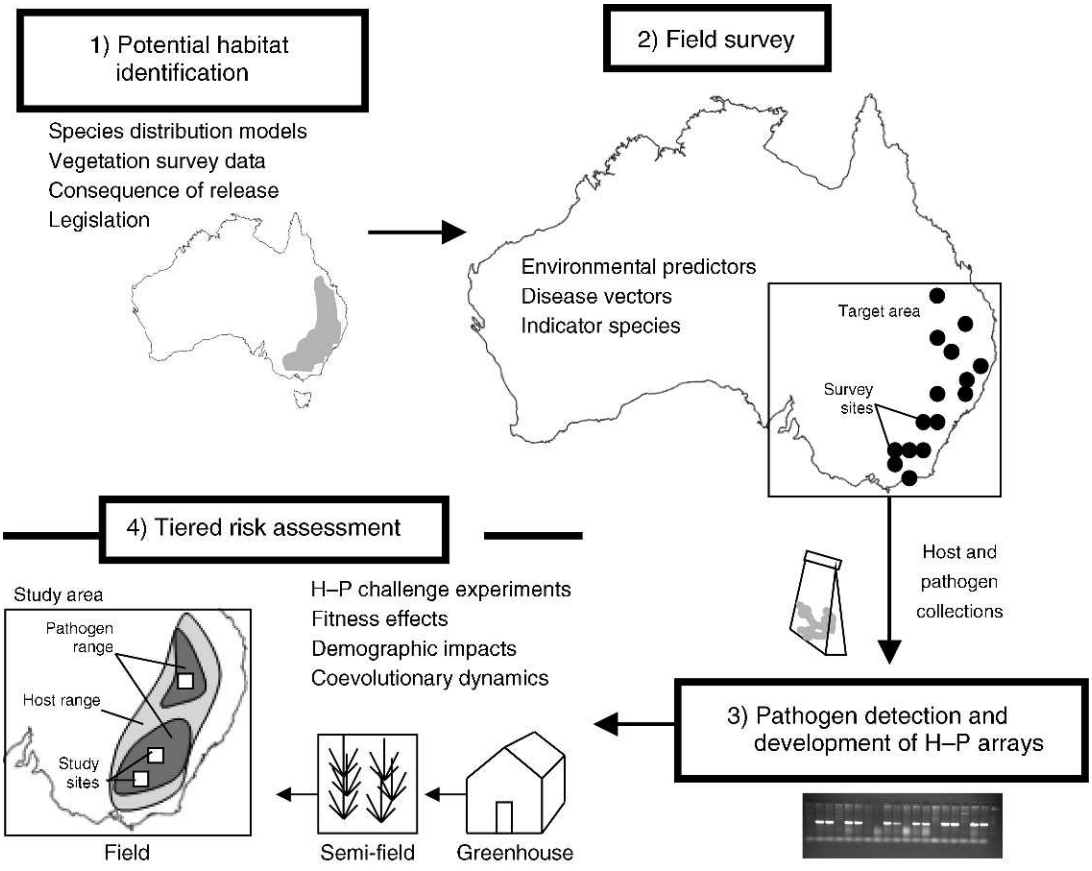

FIG. 1. A procedure for assessing the potential risk posed by pathogen-resistant plants to nontarget ecosystems at large spatial scales. Identification of potential habitats (Stage 1) and field surveys in the target area (Stage 2) are key to generation of representative host–pathogen (H–P) arrays (Stage 3). These arrays are then used in controlled H–P challenge experiments and in situ demographic field studies that target at-risk habitats (Stage 4). Details concerning the implementation of tiered risk assessment strategies are provided in Wilkinson and Tepfer (2009) and Godfree et al. (2009*a*, *b*).

WClMV–*T. repens* pathosystems, and the extent of co-infection in nontarget host populations. Our specific objectives were to:

1) determine the distribution and abundance of *T. repens* and co-existing viral pathogens (AMV, ClYVV, WClMV) in nontarget habitats (including endangered ecosystems) that occur in a range of bioregions in southeastern Australia;

2) identify the geographic and site-level factors that are associated with the distribution and abundance of *T. repens* and associated viruses in the study region;

3) determine species- and regional-level patterns of co-infection among the viral pathogens AMV, ClYVV, and WClMV;

4) identify, based on the spatial distribution of host–virus pathosystem components, nontarget habitats that may potentially be placed at risk by the release of virus-resistant *T. repens*; and to

5) understand the implications of spatial variation in pathosystem structure for development of tiered, experimental risk assessment protocols for PR plants in general.

To our knowledge this is largest study conducted to assess the ecological implications associated with the release of PR genotypes into a pathosystem complex that occurs in multiple bioregions on a continental scale. As the study required refinement of our understanding of the link between pathosystem characteristics (infection levels and distribution), habitat type (vegetation type and host abundance), land management regimes (disturbance and position within the agricultural landscape) and vector epidemiology, characteristics that are common to many natural pathosystems, our results have broad implications for the risk assessment of PR transgenic plants on a global scale.

METHODS

Selection of nontarget habitats for Trifolium repens *and virus surveys*

We selected 213 survey sites in which to assess *T. repens* abundance and virus frequency within a 300 000-km^2 study area in southeastern Australia (Fig. 2a). Sites were present in 37 potential habitat types (Table 1) that occurred within the climatic envelope for *T. repens* (Hill

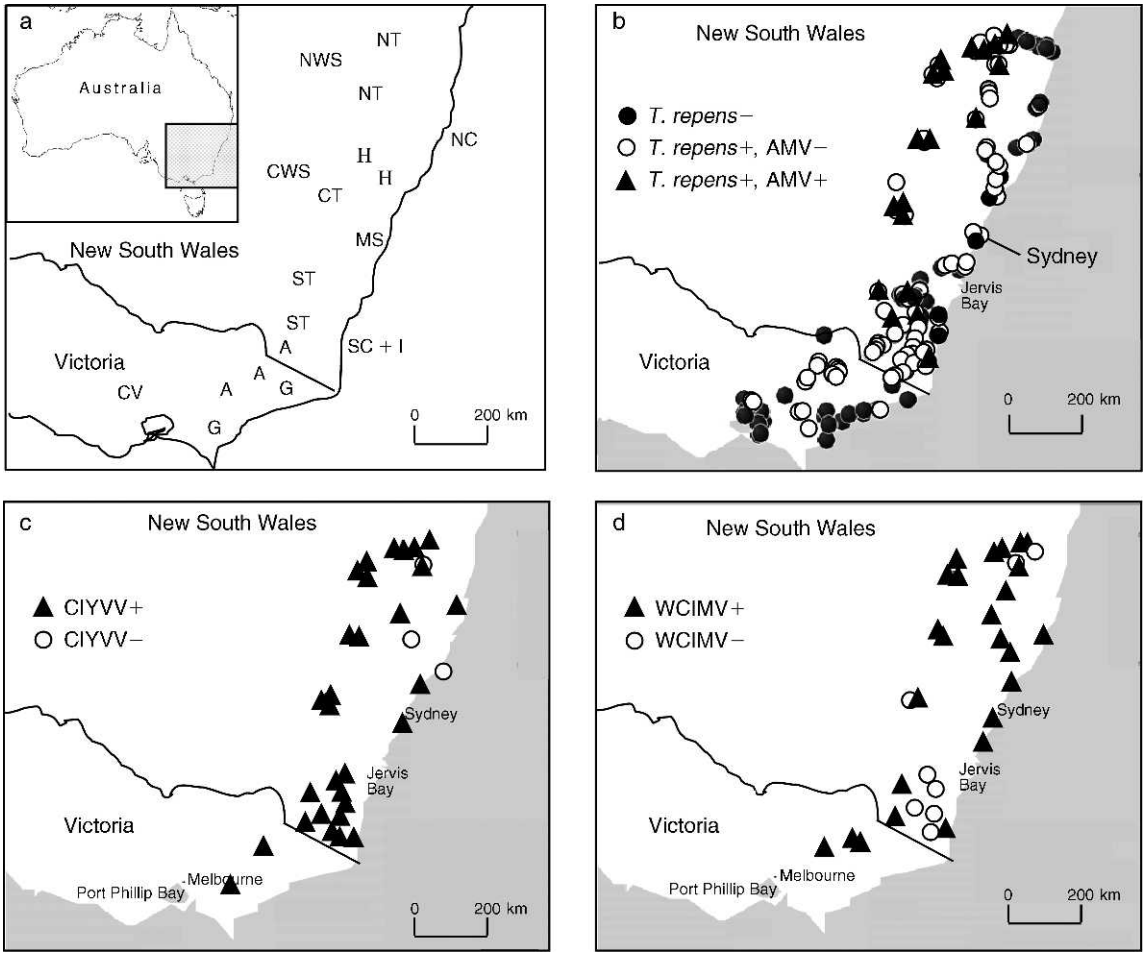

FIG. 2. Location of survey sites in southeastern Australia. (a) Geographic regions referred to in New South Wales (NSW) and Victoria; the insert shows the position of the general study region within Australia. (b) Position of all survey sites containing *T. repens* and alfalfa mosaic virus (AMV). (c) Distribution of clover yellow vein virus (ClYVV) based on sites tested for ClYVV. (d) Distribution of white clover mosaic virus (WClMV) based on sites tested for WClMV. Abbreviations on panel (a) are: NWS, northwest slopes; NT, northern tablelands; CWS, central west slopes; H, Hunter Valley and Barrington regions; NC, north coast, CT, central tablelands; MS, metropolitan Sydney; ST, southern tablelands; A, alpine and subalpine region (NSW and Victoria); SC + I, south coast and Illawarra; G, Gippsland Victoria; CV, central Victoria.

1996). Most habitats had high conservation value (listed as threatened or endangered at state or federal level), were within the region where commercial release of PR *T. repens* genotypes is likely, or were likely to contain large virus-infested populations of *T. repens* to act as a source of viral inoculum (e.g., roadsides). The list of potential habitats was determined based on consultation with the relevant literature describing the flora of southeastern Australia, identification of endangered or threatened plant communities, and consultation with relevant government bodies (e.g., the Office of the Gene Technology Regulator [OGTR]) involved in the risk assessment process. Survey sites were widely distributed across southeastern Australia (Fig. 2a) and occurred in range of geographic regions and bioregions that delineate dominant vegetation types across the study region (Table 1). Most importantly, the survey included 21 plant communities that were threatened or endangered at the national or state level, a range of communities occurring in Wetlands of National Significance (WNS), and numerous sites in National Parks (NP; Table 1). Relevant references for all habitats and regional plant communities are provided in Appendix A.

Habitat classification and Trifolium repens *abundance*

At each survey site we recorded a range of parameters for habitat description and quantification of the distribution and abundance of *T. repens* and associated viruses. These were location (latitude and longitude), habitat type (see Table 1), *T. repens* abundance, disturbance level, distance (nearest km) to closest cropped area ($\leq$1 km or $>$1 km), and conservation value. *Trifolium repens* abundance was determined based on a semi-quantitative scale containing five classes (0,

absent; 1, a few plants present, 0–1% cover; 2, common with 1–5% cover; 3, abundant, a dominant understory plant with 5–30% cover; 4, very abundant and approaching a monoculture in many areas with >30% cover overall; see Plate 1). Cropping activity in surrounding sites was defined as obvious tillage or cultivated *T. repens* pasture.

The level of disturbance at each site (at the time of sampling) was classified as high, medium, or low. Highly disturbed sites were those in which disturbance had severely limited the growth of native species and strongly altered the structure of the plant community; this usually occurred due to severe grazing by livestock, extensive fire, or land management practices that altered the physical environment. Sites characterized by medium disturbance contained partially intact native vegetation but with clear evidence of compositional and structural change; usually associated with activities such as light grazing or occasional mowing. Sites with a low disturbance ranking were characterized by minimal recent disturbance and contained largely intact native plant communities.

Conservation value of each site was classified on the basis of four subjective categories arranged in generally declining conservation significance. Categories were (1) very high (endangered or threatened plant communities listed within Australia at the state or federal level, WNS and wetlands listed under the Convention on Wetlands of International Importance [the Ramsar Convention]); (2) high (sites containing largely intact, minimally disturbed, remnant native vegetation within a NP, travelling stock reserve [TSR] or other type of reserve); (3) medium (native vegetation occurring within a NP, TSR, reserve or adjacent to a road but with a moderate to high level of disturbance); and (4) low (heavily disturbed areas and roadside verges [within 5 m of the road edge] with little or no remaining native vegetation).

In addition to the 37 primary habitat types investigated in the study (Table 1), sites from across the survey region were also grouped into the following broader habitat types that capture much of the general floristic variation present in southeastern Australian vegetation: (1) alpine bog, heath, and snow patch; (2) coastal and coastal plain forest, woodlands, and grasslands; (3) inland lowland, montane, and subalpine forests and woodlands; (4) inland lowland, montane, and subalpine grassland; (5) wetlands, swamps, and salt marshes; and 6) roadsides, heavily modified vegetation, and stock reserves (Table 1).

Trifolium repens *collections*

Trifolium repens stolons were collected between January 2006 and April 2007 from 125 survey sites where *T. repens* was present. At each site we collected up to 50 stolons (approximately 5 cm long with two or three nodes and at least 1 m apart) from a representative area that varied in size from 100 m^2 to 10 ha, depending on plant density and habitat size. Stolons were transported on ice to CSIRO Black Mountain Laboratories (35°16′23.12″ S, 149′06′49.27″ E) and planted into 5-cm pots containing sterilized compost. Plants were kept covered with clear plastic in a growth room for two weeks, and then transferred to a climate controlled glasshouse maintained at an approximately 15/25°C night/day temperature regime for further growth.

Virus detection

All sites where *T. repens* was collected were tested for AMV, however, due to resource limitations fewer sites were tested for WClMV and ClYVV. With the aim of gaining co-infection data for the study region, sites tested for WClMV and ClYVV included all sites found infected with AMV (19 sites) plus in additional set of sites (13) randomly selected from within the whole survey region (Fig. 2) for comparison. As no obvious pattern in the incidence of WClMV or ClYVV was observed between the sites infected or uninfected with AMV (Fig. 2), the number of sites sampled was considered sufficient to allow a non-biased comparison of the distribution and frequency of all three virus species.

We used three methods to identify and quantify the presence of AMV, WCIMV, and CIYVV in *T. repens*: indicator-plant bioassays, polymerase chain reaction (PCR), and immunoassays. PCR was only used to identify AMV isolates since previous evidence showed that WClMV and ClYVV could be reliably detected using bioassay techniques (Godfree et al. 2004*a*). For all tests AMV-positive sap controls were taken from *T. repens* plants collected near Canberra, Australian Capitol Territory (ACT), Australia, while sap from plants grown from seed was used as a negative control (AMV, ClYVV, and WClMV are not seed transmitted in *T. repens*; (Latch and Skipp 1987, Johnstone and Chu 1993)). Indicator-plant bioassays, in which viral identification is based in symptoms in the leaves of the indicator plants cowpea (*Vigna unguiculata*) and *Chenopodium amaranticolor* was performed as described in Godfree et al. (2004*a*).

Detection of alfalfa mosaic virus using RT-PCR and virus-specific PCR.—Two sets of AMV specific PCR primers were utilized to detect AMV in *T. repens*. Initially AMV primers developed by Bariana et al. (1994) were used but the majority of the work was performed using primers F2 and R2 as described by Xu and Nie (2006) because it was found that the original primers had homology to a region of the AMV genome where variation was known to occur. *Trifolium repens* leaf tissue (<100 mg) was submerged in liquid nitrogen, ground quickly with a cold mortar and pestle, and stored at −80°C. RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's protocol. Extracted RNA was stored in RNAse-free water at −80°C.

Samples (5 μL, 100 ng–5 μg total RNA) of extracted RNA, 5.5 μL RNAse-free H_2O, and 2.0 μL (15–20

TABLE 1 Habitat types surveyed for *Trifolium repens*, alfalfa mosaic virus (AMV), clover yellow vein virus (ClYVV), and white clover mosaic virus (WClMV).

Habitat type	ID	Region	Conservation value	Disturbance level
Alpine				
Bog community or fen (bog pool) community	1	3	VH	L,M
Heath	2	9	H	L
Snowpatch community	3	3	VH	L,M
Coastal and coastal plain				
Northern warm temperate rainforest	4	4	H	L
Bega dry grass forest	5	4	VH	L
Brogo wet vine forest	6	4	VH	L
Candello dry grass forest in the Southeast Corner bioregion	7	4	VH	H
Lower Hunter spotted gum- ironbark forest in the Sydney Basin bioregion	8	9,13	VH	L, M
South coast sands and southern tablelands dry sclerophyll forest	9	4,9	M, H	L, M, H
Southern escarpment wet sclerophyll forest remnant	10	1	VH	L
Subtropical forest	11	13	H	L
Coastal woodland and grassland	12	1,2,4,5	M, H	L, M
Inland lowland, montane, and subalpine forests and woodlands				
Montane wet sclerophyll forest	13	9	M, H	L, M
Ribbon gum, mountain gum, snow gum, grassy forest woodland of the New England tableland bioregion	14	1, 13	VH	L, M
New England peppermint woodland on basalts and sediments in the New England tableland bioregion	15	7	VH	L
Inland *Casuarina* riparian woodland	16	11	M	H
White box, yellow box, and Blakely's red gum grassy woodland, and derived native grassland	17	7,11	VH	L, M
Subalpine and montane woodland	18	3, 9, 10, 13	M, H	L, M, H
Lowland, montane, and subalpine grassland				
Central Gippsland Plains grassland community	19	1	M	M, H
Subalpine sod–tussock grassland	20	10	VH	L, M
Montane grassland	21	3	H	M
Natural temperate grassland of the southern tablelands of NSW and the Australian Capital Territory	22	9	VH	L, M, H
Plains grassland (South Gippsland) community	23	1	VH	L
Western (basalt) plains grassland community	24	2	VH	L, M, H
Wetlands, swamps, salt marshes				
Upland wetlands of the New England tablelands and the Monaro Plateau, upland wetlands of the drainage divide of the New England tableland bioregion	25	7	VH	L, H
Montane peatlands and swamps of the New England tableland, NSW north coast, Sydney Basin, Southeast Corner, Southeastern Highlands, and Australian Alps	26	3,5,9,13	VH	L, M
Fresh water wetlands on coastal floodplains of the NSW north coast, Sydney Basin, and Southeast Corner	27	4,6,13	VH	L,M
Herb-rich plains grassy wetland (West Gippsland) community (temperate lowland plains grassy wetland)	28	1	VH	H
Freshwater wetlands in the Sydney Basin bioregion	29	6	VH	M, H
Coastal salt marsh in the NSW north coast, Sydney Basin, and Southeast Corner bioregions	30	1,5	M, H, VH	L, M
Red gum swamp community	31	1,2	VH	L, M
Sedge-rich *Eucalyptus camphora* swamp community	32	9	VH	L
Coastal heath and swamp	33	1	M, H	L, M
Wetlands (other)	34	1,2,4,5,6,9	H, VH	L, M, H
Roadsides, modified vegetation, stock reserves				
Roadsides, disturbed (coastal, lower elevations)	35	2,4,5,8,9,11, 13	L, M	M, H
Disturbed, roadsides (subalpine, alpine)	36	3,9,10	L, M	M, H
Traveling stock reserves with minimal native vegetation	37	4,5,7,9	L, M, H	L, M, H

Notes: Categories for conservation value, disturbance level, cropping proximity, and *T. repens* abundance are provided in *Methods: Habitat classification and* Trifolium repens *abundance*; the main classes present in each habitat type are shown. All sites in which *T. repens* was present were tested for the presence of alfalfa mosaic virus (AMV), while a subset were tested for white clover mosaic virus (WClMV) and clover yellow vein virus (ClYVV). Regions are: 1, Gippsland Victoria; 2, central Victoria; 3, alpine and subalpine Victoria; 4, south coast and Illawarra NSW; 5, mid- and north-coast NSW; 6, metropolitan Sydney; 7, northern tablelands NSW; 8, central tablelands NSW; 9, southern tablelands NSW and ACT; 10, alpine and subalpine NSW; 11, northwest slopes NSW; 12, central west slopes NSW; 13, Hunter Valley and Barrington region, NSW.

TABLE 1 Extended.

Crop ≤1 km	No. sites surveyed	No. sites with T. repens	*T. repens* abundance	No. sites with AMV	No. sites with WClMV	No. sites with ClYVV
N	5	1	1.0	0		
N	2	2	4.0	0		
N	2	2	2.0	0		
N	2	0		0		
Y, N	6	2	1.0	0		
N	3	0				
Y	1	1	2.0	0		
Y, N	5	2	1.5	0		
Y, N	6	1	1	0	0/1	1/1
N	1	0				
N	1	0				
N,Y	10	2	2.5	0	0/1	1/1
N	3	3	4.0	0	1/1	1/1
Y, N	3	3	2.3	1	0/1	1/1
Y	1	1	4.0	1	0/1	1/1
Y	1	1	3.0	1	1/1	1/1
Y, N	7	7	3.1	2	2/3	2/3
Y, N	24	19	2.2	2	2/5	5/5
N	2	1	1	0		
N	6	6	3.7	1	2/2	2/2
N	1	1	3	0		
Y, N	14	11	3.4	2	1/3	3/3
N	1	0				
N	4	2	2	0		
Y, N	2	2	4.0	1	1/1	1/1
N	7	5	2.6	0	0/1	1/1
N	8	2	1.5	0		
Y	1	0				
N	2	0				
N	3	0				
Y, N	2	0				
Y	1	0				
Y, N	3	1	1	0		
Y, N	15	1	4	0		
Y, N	29	27	3.2	7	7/9	8/9
Y, N	14	14	3.4	0	0/1	1/1
Y, N	15	5	1.8	1	1/1	1/1

pmol) antisense primer were incubated for 5 min at 70°C in a Hybaid PCR Express (Integrated Sciences, Chatswood, New South Wales, Australia). Samples were held at 4°C while 4 μL 5× Reaction Buffer (MBI Fermentas RevertAid, Burlington, Ontario, Canada), 2 μL 5 mmol/L dNTPs (final concentration of 1 mmol/L) and 0.5 μL RNAsin (Promega inhibitor; Promega, Madison, Wisconsin, USA) was added and incubated 5 min at 37°C then held at 4°C while 1 μL (200 units) of M-MuLV Reverse Transcriptase (MBI Fermentas RevertAid) was added. Samples were then incubated for 60 min at 42°C, 10 min at 70°C, and then incubated on ice if used immediately; otherwise samples were stored at −20°C.

Samples containing 5 μL of RT-PCR reaction, 5 μL 10× PCR Reaction buffer (Perkin Elmer, Waltham, Massachusetts, USA), 3 μL $MgCl_2$ (Perkin Elmer), 2 μL 5 mmol/L dNTPs, 1 μL Forward primer (250 ng/μL), 1 μL Reverse primer (250 ng/μL), 0.5 μL Taq polymerase (Perkin Elmer), and RNAse-free H_2O to a total volume of 50 μL were then treated according to the temperature regime described by Xu and Nie (2006). PCR products were visualized by separation on a 0.7% agarose gel run at 100 V for approximately 45 min with GeneRulerTM 1 Kb Plus DNA Ladder as a standard (Fermentas). The gel was stained with ethidium bromide and bands were visualized under UV light.

Viral detection based on immunoassay.—The immunoassay method used was based on the procedure described in Graddon and Randles (1986) with AMV antibodies provided J. W. Randles (Adelaide University, South Australia, Australia). Clover yellow vein virus and WClMV antibodies were provided by Paul W. G. Chu (CSIRO Plant Industry, Canberra, Australian Capital Territory, Australia).

Trifolium repens leaf tissue (<0.5 g) was placed in a plastic bag with an equal volume (w/v) of phosphate buffered saline (PBS; 0.14 mol/L NaCl, 1.5 mmol/L KH_2PO_4, 8.1 mmol/L Na_2HPO_4, 2.7 mmol/L KCl, pH 7.4) and crushed. The supernatant was transferred to a 1.5-mL Eppendorf tube and centrifuged at 10 000 g for 2 min. The supernatant was applied to nitrocellulose membrane in a series of 1-μL samples and air dried. The membrane was stored between filter paper at −20°C. The membrane was blocked by immersion in blocking buffer B containing PBS, 2.6% skim milk powder, and 10% supernatant from healthy *T. repens* (2 g healthy leaf tissue crushed with an equal volume (w/v) of PBS and spun at 3000 rpm for 5 min) to absorb non-viral antibodies and was incubated for 15 min at 37°C with gentle shaking. The buffer was discarded, replaced with AMV-, ClYVV-, or WClMV-specific antibody diluted 1/1000 in blocking buffer B (PBS, 2.6% skim milk powder, 10% supernatant from healthy leaves) and incubated for 30 min at 37°C with shaking. The membrane was washed in Blocking buffer A (PBS, 2.6% skim milk powder) three times for three min. The buffer was discarded and the membrane immersed in alkaline phosphatase conjugated goat anti-rabbit gamma-globulin (Sigma Chemicals, Perth, Western Australia, Australia) diluted 1/1000 in PBS containing 1% bovine serum albumin (BSA) and incubated for 30 min at 37°C with shaking. The nitrocellulose membrane was washed twice for three min in AP 7.5 (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 2 mmol/L $MgCl_2$, 0.05% Triton X100, pH 7.5) and twice for three min in AP 9.5 (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 5 mmol/L $MgCl_2$, pH 9.5) at room temperature. The wash was discarded and the substrate BCIP/NBT-Blue Liquid Substrate System for Membranes (Sigma-Aldrich, St. Louis, Missouri, USA) added. The membrane was incubated at low light intensity with shaking until blue dots (positive samples) appeared. The substrate was drained and the stop buffer (10 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 7.5) added.

Statistical analyses

Distribution of Trifolium repens *and associated viruses*.—Patterns of *T. repens* occurrence were compared across broad habitat type (Table 1), conservation class (very high, high, medium, low), disturbance level (high, medium, low) and proximity to cropping ($\geq$ 1 km vs. $<$ 1km) using Pearson's χ^2 goodness-of-fit test (Sokal and Rohlf 1987). Contingency tables were constructed based on the total numbers of sites with and without white clover that occurred in each predictor variable category. We also used one-way ANOVA to compare the mean abundance of *T. repens* plants at each site across broad habitat type, disturbance level, and proximity to cropping classes; post hoc means tests were performed using the Tukey-Kramer correction for multiple testing (Sokal and Rohlf 1981).

We compared the pattern of AMV presence across conservation, disturbance, and crop proximity classes based on the numbers of sites in which each virus was present or absent; only sites containing white clover were included in total site counts in each classification category. Pearson's χ^2 was used to test goodness of fit (Sokal and Rohlf 1987) unless >25% of expected cell counts were $<$ 5, or if at least one cell had an expected count of <1, in which case we used Fisher's exact test (FET; Sokal and Rohlf 1987). In practice, both χ^2 and FET provided similar results.

Single- and co-infection among AMV, C1YVV, and WC1MV.—We investigated associations among AMV, ClYVV and WClMV based on 365 plants collected from 13 sites (11–81 plants per site) across the study area in which all three virus species had been detected. We first determined the percentage of plants containing single (AMV+, ClYVV+, WClMV+), double (AMV+/ClYVV+, AMV+/WClMV+, WClMV+/ClYVV+), and triple (AMV+/ClYVV+/WClMV+) co-infections, then tested for association among virus species using log linear analysis of the associated three-way ($2 \times 2 \times 2$) contingency table (Tabachnick and Fidell 1996) containing numbers of plants in each co-infection class. We compared saturated and unsaturated models (containing no three-way interaction) using the likelihood-ratio test

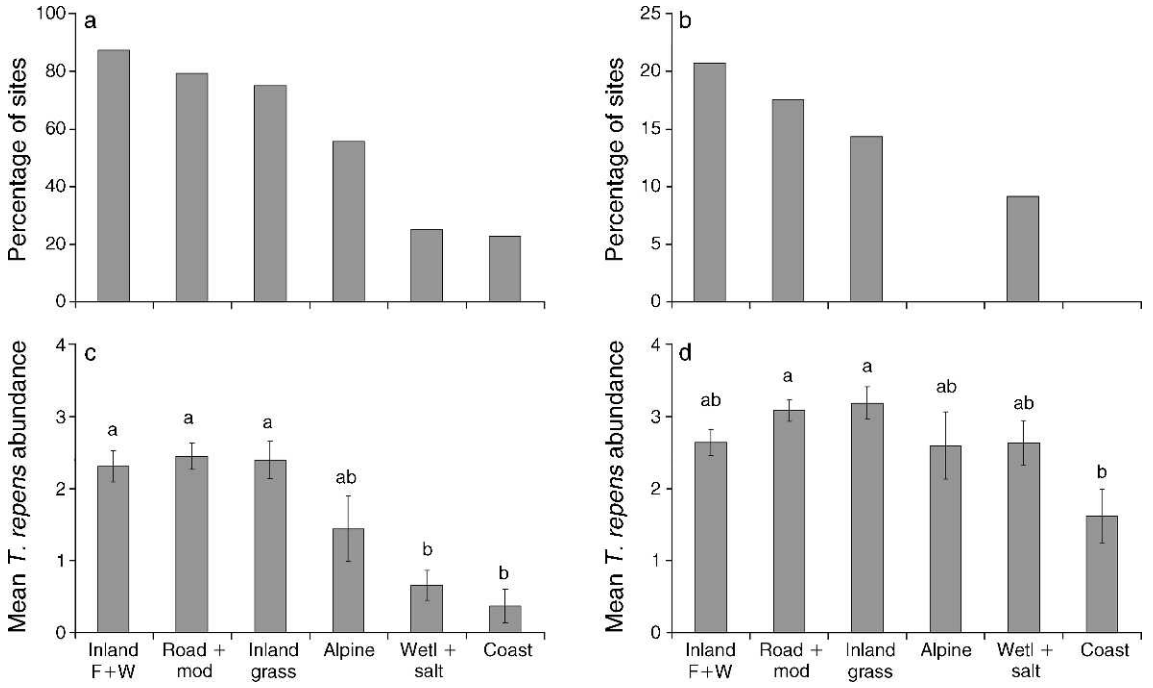

FIG. 3. (a) Percentage of surveyed sites containing *Trifolium repens* in each of the six main vegetation types; (b) percentage of *T. repens* sites containing alfalfa mosaic virus (AMV); (c) mean abundance (±SE) of *T. repens* across all sites in each vegetation type; (d) mean abundance (±SE) of *T. repens* in sites that contained *T. repens* (i.e., excluding sites where *T. repens* was absent). In panel (c), differences among classes were significant ($F_{5,207} = 18.0$, $P < 0.001$); in panel (d), differences were also significant ($F_{2,207} = 4.0$, $P < 0.01$). In panels (c) and (d), means sharing the same letter above the histogram bars are not significantly different at the $P < 0.05$ level. Vegetation types are: Inland F+W, inland lowland, montane, and subalpine forests and woodlands; Road + mod, Roadsides and modified vegetation on stock reserves; Inland grass, inland lowland, montane, and subalpine grassland; Alpine, alpine bog, heath, and snowpatch; Wetl + salt, wetlands, swamps, and salt marshes; Coast, coastal and coastal plain forests, woodlands, and grasslands.

statistic (G); model parameters were estimated using the maximum likelihood method. Since the results of log linear analysis indicated the presence of a three-way interaction term among the virus species, we then tested for the presence of each pair of viruses split by presence or absence of the third virus; again using the likelihood ratio test statistic (G) as the test of association.

We used the same procedure to test for regional differences in association among the three viruses based on plants collected from four sites in central New South Wales, Australia (NSW, $n = 65$ plants) and five sites in northern NSW ($n = 131$ plants) where AMV, ClYVV, and WClMV were all present. For the central NSW sites log-linear analysis of the AMV × ClYVV × WClMV contingency table indicated the presence of a significant three-way interaction ($G_{(1)} = 6.9$, $P < 0.01$) and so we separately assessed relationships between each virus pair in the presence and absence of the third virus. For the northern site, we used backward selection to select the most parsimonious model, and a supplementary G test of association was made on the AMV × ClYVV interaction with cell counts averaged over both WClMV classes (+/−).

All contingency analyses were conducted using SAS Proc Genmod and Proc Freq version 9.1 while GLM analyses were conducted using SAS Proc GLM version 9.1 (SAS Institute 2003).

RESULTS

Distribution, community affinity, and abundance of Trifolium repens

Trifolium repens was found in 125 (59%) of the 213 sites surveyed in the study, (Fig. 2, Table 1). There was a distinct trend for *T. repens* to be most prevalent in cool, high altitude tableland and alpine regions of NSW, Victoria, and the Australian Capital Territories (ACT; Fig. 2b) and along roadsides in the drier and warmer western slopes and Hunter Valley regions of NSW, but to be scattered or rare in coastal areas (Fig. 2b). Reflecting this geographic distribution, *T. repens* occurred less frequently in coastal and wetland habitats (≤ 25% of sites) than in inland grasslands, forests and woodlands and in roadsides and other highly modified areas (≥ 75% of sites; $\chi^2_{(5)} = 65.5$, $P < 0.001$ across all habitat types; Fig. 3a). *Trifolium repens* occurred in an intermediate percentage of alpine sites (Fig. 3a). Similarly, mean *T. repens* abundance across all surveyed sites was highest in inland and modified habitats (average abundance score > 2, Fig. 3c) and lowest in coastal and wetland habitats (Fig. 3c). These differences

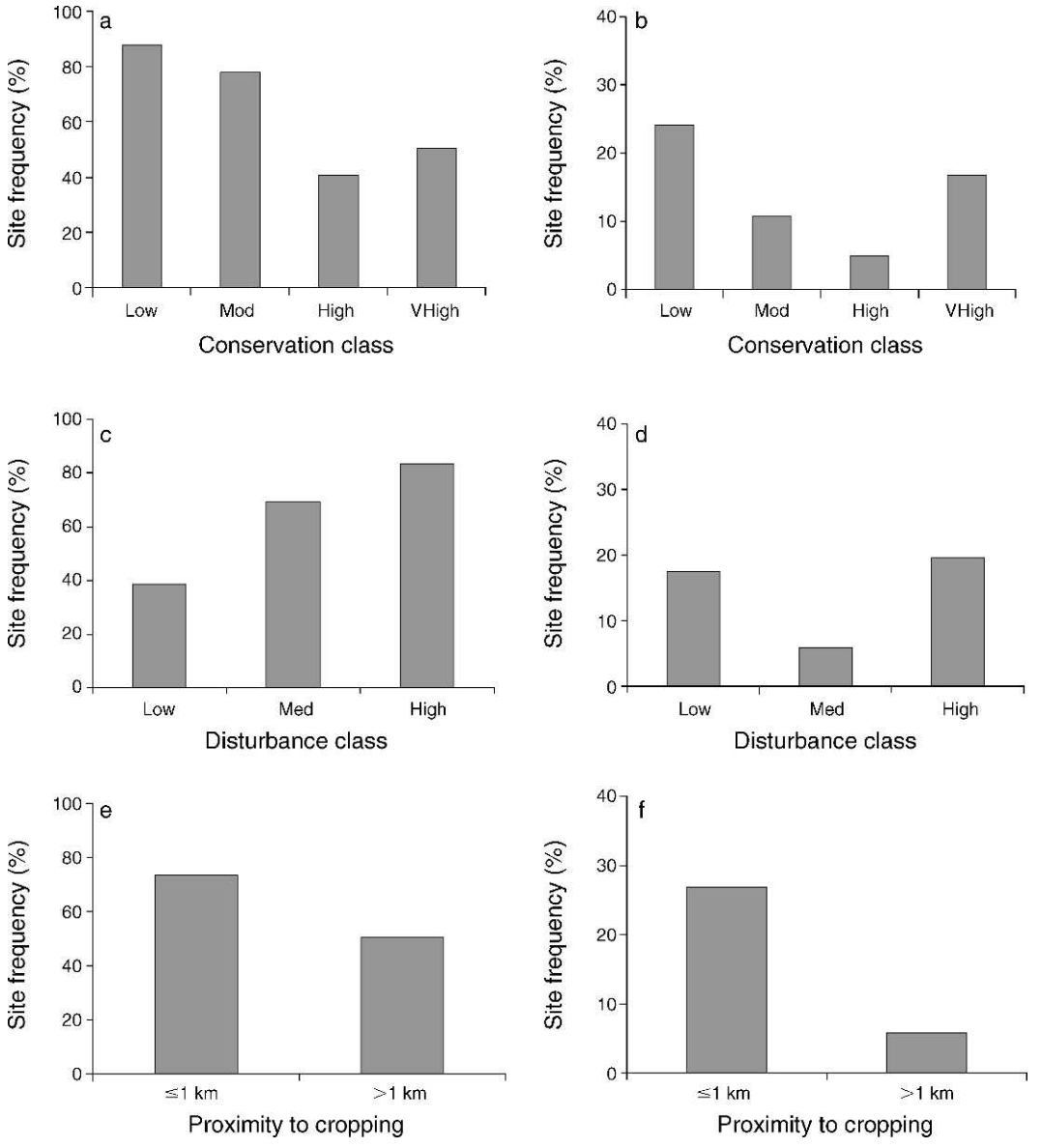

FIG. 4. Factors influencing the presence of *Trifolium repens* and alfalfa mosaic virus (AMV) in southeast Australia. (a) Percentage of surveyed sites containing *T. repens* in each of the four conservation classes (low, moderate, high, very high); (b) percentage of *T. repens* sites containing AMV in each conservation class; (c) percentage of sites containing *T. repens* in each disturbance class (low, medium, high); (d) percentage of *T. repens* sites containing AMV in each disturbance class; (e) percentage of sites containing *T. repens* in relation to crop proximity; (f) percentage of *T. repens* sites containing AMV in relation to crop proximity. All classification systems are described in *Methods: Habitat classification and* Trifolium repens *abundance*.

were less strong when compared over only those sites that contained *T. repens* (i.e., abundance scores of ≥ 1; Fig. 3d), but where present, coastal *T. repens* populations were less abundant than those present in modified and inland grassland habitats.

Of the 37 primary potential habitat types investigated in the study, 27 (73%) contained *T. repens* (Table 1). These included a wide range of native plant communities along with roadsides, revegetation sites, and travelling stock reserves (TSRs), but, most importantly, included a number of very high conservation-value, nationally or federally listed, endangered native plant communities (see Plate 1). Invasion levels were especially high in endangered natural temperate grasslands in NSW and the ACT (11 of 14 surveyed sites [Carter et al. 2003, Environment ACT 2005]) and subalpine and montane woodlands (19 out of 24 sites). *Trifolium repens* was nonrandomly distributed across conservation classes ($\chi^2_{(3)} = 26.1$, $P < 0.001$; Fig. 4a), with sites of low and moderate conservation value having a higher rate of infestation (88% and 78% of sites, respectively) than high and very high conservation-value sites (41% and

51%). Mean (± SE) site abundance of *T. repens* followed a similar pattern (2.58 ± 0.27, 2.19 ± 0.26, 1.16 ± 0.26, and 1.40 ± 0.16 for conservation classes low, medium, high, and very high, respectively; $F_{3,209} = 7.8$, $P < 0.001$). However, these differences were due to differences in the frequency of sites containing *T. repens*, since mean abundance did not differ across those sites that contained *T. repens* (i.e., a minimum site abundance of 1 or more; $F_{3,121} = 0.1$, $P = 0.94$).

Trifolium repens presence was positively associated with site disturbance score ($\chi^2_{(2)} = 34.7$, $P < 0.001$), with 83% of highly disturbed sites containing *T. repens* compared with 69% and 39% of sites with medium and low levels of disturbance, respectively (Fig. 4c). Significant differences in abundance means ($F_{2,210} = 15.0$, $P < 0.001$) across disturbance categories (mean ± SE = 1.16 ± 0.15, 1.67 ± 0.22, and 2.51 ± 0.20 for low, medium, and high, respectively) primarily reflected differences in infection frequency, although among only sites that contained *T. repens* ($n = 125$) there was a weak trend ($F_{2,122} = 3.6$, $P = 0.03$) for sites with medium disturbance to have a lower abundance (2.41 ± 0.18) than sites with low (2.98 ± 0.17) or high (3.00 ± 0.15) disturbance. *T. repens* also tended to occur more frequently in sites within 1 km of cropped land (74% of sites) than in those >1 km away (50% of sites; $\chi^2_{(1)} = 11.0$, $P < 0.001$; Fig. 4e), and also had a higher mean abundance of *T. repens* (1.97 ± 0.19 vs. 1.49 ± 0.14; $F_{1,211} = 4.4$, $P = 0.04$). This difference in means, which explained only 2% of variation in the data (model $R^2 = 0.02$), was not significant when sites that contained no *T. repens* were removed ($F_{1,123} = 2.0$, $P = 0.16$).

Prevalence of viruses in Trifolium repens *populations*

AMV was detected at 19 of 125 sites (15%) that contained *T. repens* (based on at least two methods of detection), and occurred in 7% to 95% (46% site average, 31% of plants overall) of white clover plants collected in these sites. The location of these sites is shown in Fig. 2b; no AMV was found in Victoria during the survey, and there was a strong tendency for AMV to infest white clover populations growing in northern NSW (Fig. 2b).

AMV presence was not associated with broad habitat type ($P = 0.80$, FET; Fig. 3b), but it was present in eight moderate to very high conservation value communities (Table 1), most notably lowland temperate grasslands of southern NSW and the ACT, white box, yellow box, and Blakely's red gum grassy woodlands and derived native grasslands, New England peppermint woodland on basalts and sediments in the New England tableland bioregion, and montane peatlands and swamps (Table 1).

There was no evidence for a difference in the proportion of *T. repens* sites containing AMV among all four conservation value classes ($P = 0.30$, Fisher's exact test, FET; Fig. 4b), or among disturbance classes ($\chi^2_{(2)} = 3.2$, $P = 0.20$; Fig. 4d). AMV was more likely to be found in sites close to (≤1 km) cropping or agricultural *T. repens* pastures ($\chi^2_{(1)} = 10.6$, $P < 0.01$; Fig. 4f), and was positively associated with *T. repens* abundance ($\chi^2_{(2)} = 8.0$, $P = 0.02$) across the three abundance classes (classes 1+2, 3, and 4). This reflected the much greater prevalence of AMV in sites containing the most abundant *T. repens* populations (class 4 = 5/39 (13%) of sites; class 3 = 13/52 (25%) of sites compared with sites with low *T. repens* abundance (combined classes one and two; 1/33 or 3% of sites).

Although all plants collected were tested for AMV, only plants from 32 sites were also tested for ClYVV and WClMV. Clover yellow vein virus was detected in 30 sites (94%) at an average site infection rate of 36% (range 3–100%; 31% of plants infected overall). White clover mosaic virus was detected in 18 sites (56%) with an average percentage of infected plants of 30% (range 1–89%; 14% of plants infected overall) in infested sites. Information regarding the sites infested with WClMV, ClYVV, or AMV is presented in Table 1.

Co-infection of Trifolium repens

Of the 365 plants taken from surveyed sites in which all three viruses were present (AMV, WClMV, and ClYVV), 74% were infected by at least one virus, and 47%, 41%, and 19% were infected with AMV, ClYVV, and WClMV, respectively. Overall, 46% of plants were infected by only one virus (19% with ClYVV, 7% with WClMV, and 20% with AMV), 24% were infected with two viruses (6% with AMV + WClMV, 17% AMV + ClYVV, and 1% ClYVV + WClMV) and 4% were infected with all three viruses (Fig. 5a). Among plants infected with AMV, 42% contained no other viruses, while the remaining 58% were also infected by ClYVV, WClMV, or both.

Significant differences were evident in the abundance of AMV, ClYVV, and WClMV at the regional scale (Fig. 5b–c). Alfalfa mosaic virus was far more prevalent in northern NSW, where it occurred in 67% of plants, compared with central NSW (33%). White clover mosaic virus was much less common in northern NSW (18% vs. 30% of plants), while ClYVV tended to be more common (31% vs. 21%). Overall, co-infected plants were more frequent in central NSW than northern NSW (52% vs. 42% of infected plants), while 70% of AMV-infected plants also contained ClYVV, WClMV, or both in central NSW, compared with 50% in northern NSW.

Loglinear analysis of the distribution of AMV, ClYVV, and WClMV infection across the 365 tested plants indicated the existence of significant interactions between AMV, ClYVV, and WClMV ($G_{(1)} = 6.00$, $P = 0.01$), with the association between any two viruses dependent on the presence or absence of the third virus. Analysis of 2 × 2 contingency tables showed that plants co-infected by both AMV and ClYVV appeared together significantly ($G_{(1)} = 8.47$, $P < 0.01$) more frequently than expected in the presence of WClMV (16/68 plants observed vs. 10.9/68 plants expected, a 7.5% increase as a proportion of the total plant number), as

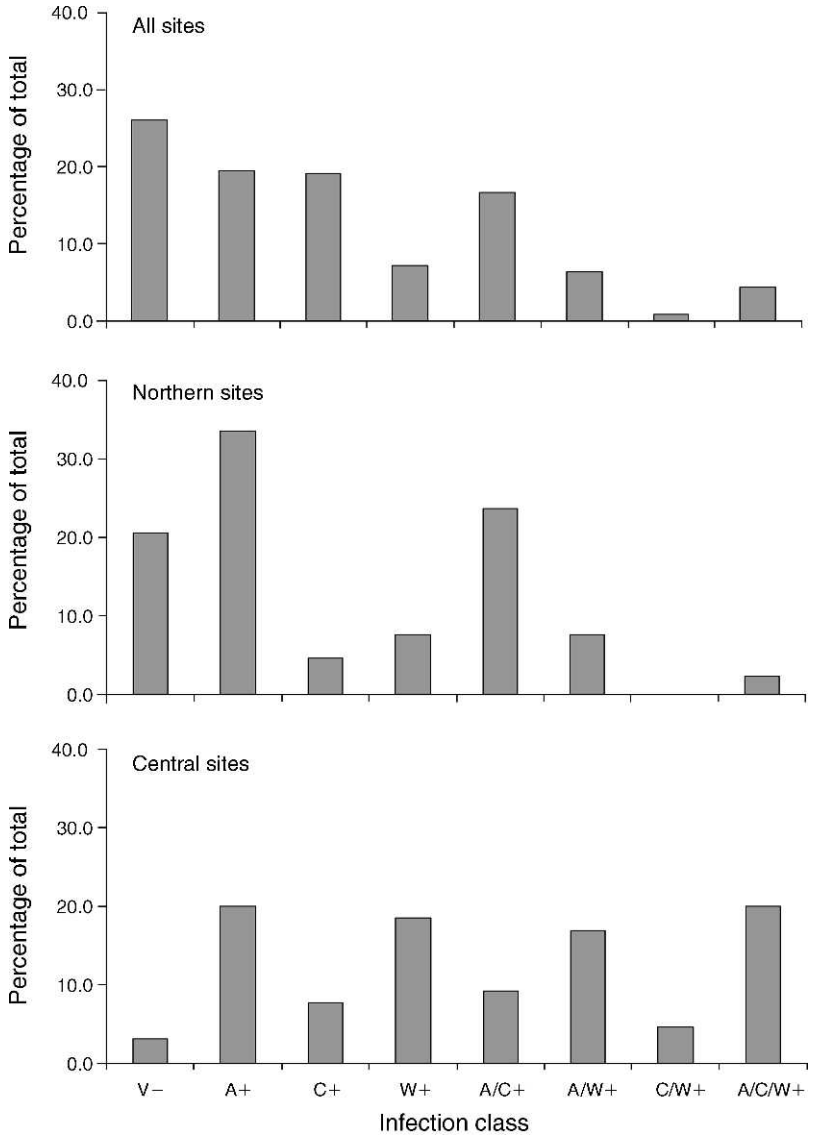

FIG. 5. Co-infection frequencies among alfalfa mosaic virus (AMV), clover yellow vein virus (ClYVV), and white clover mosaic virus (WClMV) across (a) 315 plants from 13 sites across New South Wales (NSW); (b) 131 plants from five sites in northern NSW; and (c) 65 plants from four sites in central NSW. Classes are V–, virus-free; A+, AMV+; C+, ClYVV+; W+, WClMV+; A/C+, AMV+ and ClYVV+; A/W+, AMV+ and WClMV+; C/W+, ClYVV+ and WClMV+; A/C/W+, AMV+, ClYVV+, and WClMV+.

did AMV and WClMV in the presence of ClYVV (+4.1%, $G_{(1)} = 10.3$, $P < 0.01$). ClYVV and WClMV tended to be negatively associated in the absence of AMV (–4.1% as a proportion of the total plant number, $G_{(1)} = 12.7$, $P < 0.001$). All other two-way virus interactions were not significant ($P > 0.05$).

At sites in central NSW, the three-way AMV × ClYVV × WClMV interaction was significant ($G_{(1)} = 6.88$, $P < 0.01$), and so all two-way interactions were assessed. AMV and ClYVV were positively associated when WCMV was present ($G_{(1)} = 4.7$, $P < 0.05$), while AMV and WClMV, and ClYVV and WClMV, were negatively associated in the absence of ClYVV and AMV, respectively ($P < 0.05$ for both). Finally, at the northern sites AMV and ClYVV were positively associated ($G_{(1)} = 9.1$, $P < 0.01$), with co-infected plants occurring in around 5% more plants (of the total sampled) than expected by chance.

DISCUSSION

Distribution and abundance of Trifolium repens *and associated viruses in SE Australia*

The aim of this study was to complete the first stages (Stages 1 and 2 in Fig. 1) of the risk assessment of genetically modified (GM) virus-resistant *Trifolium repens* in southeastern (SE) Australia, with a focus on determining the habitat-level potential for increased

PLATE 1. Little Llangothlin Montane Lake in the Northern Tablelands, New South Wales, Australia, is one of the very high-conservation-value (Ramsar, Wetland of National Significance and endangered) habitat types that is heavily invaded with *Trifolium repens* (abundance score of 4). White clover mosaic virus, clover yellow vein virus, and alfalfa mosaic virus were also found infecting *T. repens* at the site. Shown here is the dense *T. repens* population flowering on the foreshore of the lake. Photo credit: R. C. Godfree.

weediness of nontarget populations following the release of clover yellow vein virus (ClYVV), white clover mosaic virus (WClMV), and especially alfalfa mosaic virus (AMV) resistant *T. repens* genotypes. Our procedure involved the identification of potentially at-risk nontarget habitats within the study region and a subsequent large-scale survey of *T. repens* and associated viruses (AMV, ClYVV, and WClMV). This was the first such survey performed in Australia. While the prevalence of viruses in *T. repens* populations in Australia has been well documented in agricultural settings (Norton and Johnstone 1998), the range of native plant communities and other non-agricultural habitats containing this pathosystem remain largely unknown (Godfree et al. 2004*a*).

Trifolium repens plants were detected at 59% of the surveyed sites in SE Australia, indicating that *T. repens* poses an ongoing invasion risk to many of the communities and habitats investigated. Furthermore, the survey was conducted between January 2006 and April 2007, during one of Australia's worst recorded droughts (Murphy 2007), when most sites, especially in the southern part of the study region, were extremely dry; it is likely that in more favorable seasons *T. repens* would be even more prevalent. Nevertheless, our data show that *T. repens* inhabits a very diverse range of plant communities ranging from low to very high conservation value, and despite favoring mesic disturbed areas, is not restricted to any particular habitat type. Indeed *T. repens* occurs widely in mesic areas, river flats, woodlands, grasslands, and mid-to-high-altitude alpine and subalpine dry sclerophyll or grassy woodlands (Table 1) across the entire 300 000-km^2 study region. These results indicate that to effectively complete the ecological risk assessment of transgenic virus-resistant *T. repens*, potential impacts need to be considered for numerous nontarget community types across SE Australia, the implications of which are discussed below.

Besides being widely distributed, *T. repens* was abundant or very abundant at 67% of sites surveyed, with moderate and low levels of abundance observed at only 5% and 21% of sites, respectively. If we assume that

the impact of *T. repens* invasion on native plant communities is directly related to *T. repens* density, communities that may be at elevated risk of ecological damage include a range of endangered temperate grasslands, wetlands and grassy woodlands, wet sclerophyll forests, alpine and subalpine vegetation, and disturbed habitats. The distribution and ecological characteristics of these communities, and of *T. repens* indicate that the array of native plant communities invaded by *T. repens* is at least partially limited by climatic conditions or a "climatic envelope," a finding also supported by Hill (1996), and so any shifts in climatic zones due to climate change (Thomas et al. 2004) are likely to affect the regions and types of communities where *T. repens* is naturalized. Indeed, shifts in climatic zones could facilitate the invasion of *T. repens* into new areas, including endangered native plant communities. Consequently, any significant shift in the distribution of *T. repens* in the landscape would necessitate reappraisal of early stages of the risk assessment process.

A crucial element of the risk assessment process was to determine the distribution and abundance of AMV, WClMV, and ClYVV within wild *T. repens*, and to investigate the factors that influence virus distribution in the landscape. Our data show that of the sites that contained *T. repens*, 15% contained infestations of AMV, with 7–95% (mean = 46%) of plants infected at individual sites (Table 1). WClMV and ClYVV had similar infection rates (averaging 30% and 36% of plants per site respectively) to AMV (46%), but overall, both species appear to be more widespread (present in 56% and 94% of sites tested respectively) than AMV, which was most common in northern inland NSW (Fig. 2a, b). This is consistent with other studies that have shown ClYVV to be an especially widespread virus of *T. repens* populations in native plant communities in Australia (Godfree et al. 2004*a*).

The cause of these different distributions is difficult to explain, but could reflect the distribution of aphid vectors (both AMV and ClYVV are transmitted by aphids in a non-persistent manner (Latch and Skipp 1987, Johnstone and Chu 1993), variation in disturbance (WClMV is dispersed by mechanical means and often occurs in mown areas; Johnstone and Chu 1993), differences in the specificity of aphid vectors (Wang et al. 2006), transmission efficiency (Moreno et al. 2005), variation in viral titer in host plants (Martín and Elena 2009), or perhaps in the resistance of local *T. repens* genotypes to extant virus genotypes (Godfree et al. 2009a). Chronic drought in the southern part of the survey region (Murphy and Timbal 2008) could perhaps be important. Irrespective of the mechanism, however, our data show that inferring viral distributions based on vector transmission mode or distribution of the plant host is unlikely to be reliable in multi-species pathosystems: detailed surveys are clearly required.

On the other hand, we were able to identify a range of site-level factors related to the distribution of AMV and *T. repens* in the landscape. The most important drivers of *T. repens* presence or absence that we measured appeared to be habitat type, position in the agricultural landscape (proximity to crops), and disturbance level, while AMV occurred more frequently in sites with high host-population sizes lying in close proximity to crops. Virus presence is likely to be associated with reservoirs of infection nearby and environmental conditions conducive to the development of large aphid populations or other dispersal mechanisms (Minks and Harrewijn 1987), which probably in part explains why AMV was more likely to be present in large *T. repens* populations. AMV may also be more likely to survive environmental or demographic variability when host densities are high, a well-known phenomenon in host–pathogen metapopulation dynamics (e.g., Denny and Guy 2009).

The landscape matrix is also clearly a crucial factor in determining the presence/absence of AMV in wild *T. repens* populations, and in particular the presence of nearby potential sources of inoculum. We often observed that *T. repens* was more likely to be infected with AMV when lucerne (*Medicago sativa*), a key host of AMV (Jones 2004), was growing within 1 km of a given site. Indeed, in NSW there are at least 25 plant families that contain alternative host species for AMV (Appendix C; Hull 1969, National Herbarium of New South Wales 2009). Many of these hosts are introduced species that occur in agricultural landscapes, along roadsides or in disturbed plant communities, which may explain the tendency for AMV to occur in such habitats (Table 1). Collectively these results indicate that future stages of the risk assessment process should focus on high conservation value habitats with a history of disturbance, large *T. repens* populations, and sites in close proximity to agricultural land containing hosts for AMV.

Another interesting result of this work has been to identify the prevalence of co-infected host *T. repens* plants at the landscape scale. Overall, co-infected plants (containing two or three viruses) made up 38% of infected plants, however in certain regions this figure was much larger: in central NSW for example 52% of infected plants were co-infected with multiple viruses. Indeed, between 42% and 70% of AMV-infected plants also contained ClYVV, WClMV, or both, and virus species (especially AMV and ClYVV) tended to co-infect host plants together more frequently than expected by chance, perhaps reflecting the aphid-borne transmission of both species (Latch and Skipp 1987, Johnstone and Chu 1993). There is strong evidence that the presence of multiple viruses may alter virus accumulation and localization within the host, virus transmission and often result in disease synergism (Wintermantel et al. 2008, Alves-Júnior et al. 2009, Malapi-Nelson et al. 2009, Tatineni et al. 2010).

Consequently, the dynamics of viral co-infection are likely to be important in this, and many other, multi-disease pathosystems.

Many publications recommend the use of "tiered risk assessment" for the risk assessment of transgenic plants (Wilkinson et al. 2003). Briefly, the process begins with the first tier that tests the "worst case scenario" under controlled conditions such as the lab or glasshouse. If results indicate that harm/exposure is negligible, then it can be concluded that risks are negligible. However, if there is concern regarding risk following tier-one tests, then tier-two studies are conducted. Tier-two studies assess risk under more realistic conditions such as field trials. Again if harm/exposure is not demonstrated to be negligible then tier three studies are undertaken (large-scale field trials; Wilkinson and Tepfer 2009). Results gained from this process alone (Fig. 1, Stages 3–4) for transgenic AMV-resistant *T. repens* would have been limited without the process of habitat identification and a large-scale field survey undertaken prior to the tiered assessment (Fig. 1, Stages 1 and 2). We argue that without these two additional steps in the risk assessment process the complexity of this pathosystem would not have been revealed.

Implications for risk assessment of virus-resistant Trifolium repens *in SE Australia*

This study, which completes the first stages (Stages 1 and 2 in Fig. 1) of the environmental risk assessment of virus-resistant *T. repens*, shows that *T. repens* is a common weed in a broad range of environments in SE Australia and that AMV is present in a minority (15%) of invaded sites. The *T. repens*–AMV pathosystem is most prevalent in northern NSW but is apparently absent in central and eastern Victoria (at least in the habitats studied). Other viruses targeted by pathogen-resistant (PR) *T. repens* genotypes (ClYVV and WClMV) are more widely distributed (>50% of sites tested) but associated host populations usually have similar overall infestation rates. As such, the movement of genes conferring resistance to any of these virus species from commercially grown *T. repens* genotypes to nontarget host populations could potentially lead to increased weediness of *T. repens* in a wide range of threatened plant communities in southeastern Australia. To date, the magnitude of this effect has only been estimated for ClYVV in two plant community types (Godfree et al. 2009*b*).

AMV is a significant pathogen of *T. repens*, reducing growth by up to 33% (Latch and Skipp 1987). At some sites, we found AMV infestation at a frequency (>90% of plants infected) where an impact on *T. repens* population dynamics therefore seems likely. Such frequencies are higher than those observed for ClYVV sat any site in previous surveys (Godfree et al. 2009*b*). On the other hand, infection rates at some sites are sufficiently low (<10%) that a major impact on *T. repens* populations is unlikely, although we cannot rule out the possibility that low AMV infection rates may reflect high virus-induced *T. repens* mortality. AMV is known to cause death in some other plant species (e.g., Latham et al. 2004), but no studies that we are aware of have demonstrated *T. repens* mortality linked to AMV infection in the field (Gibson et al. 1981). Furthermore, our study demonstrated that AMV presence was positively associated with *T. repens* abundance in the region surveyed. This may reflect the fact that AMV is non-persistently transmitted by aphids (Latch and Skipp 1987, Johnstone and Chu 1993), and so high host density is likely to facilitate greater AMV transmission between plants. High host density may also enhance vector survival and population size, in turn increasing host infection rates.

Given that *T. repens* was abundant or very abundant at 18 of the 19 of sites infested with AMV (Appendix B), any impact on naturalized *T. repens* populations is, therefore, likely to result in an impact also on the native plant community. The next stage of the risk assessment process (Stage 4, Fig. 1) will focus on determining whether AMV resistance confers a fitness advantage to *T. repens* plants (the enemy-release hypothesis [Keane and Crawley 2002]), and whether the size of wild clover populations are being limited by AMV. If so, AMV-resistant *T. repens* would potentially pose a real risk to native plant communities in SE Australia. Similar studies would be necessary to resolve the level of risk associated with ClYVV- or WClMV-resistant genotypes.

General implications for risk assessment of disease-resistant plants

The results of this study have broader implications for the risk assessment of disease resistant plants that target single or multi-disease pathosystems. Risk assessments of plants expressing resistance to multiple pathogens must consider the distribution of all pathogens targeted, and assumptions cannot be made regarding distribution even if pathogens share a vector. We found that although AMV and ClYVV are both dispersed by aphid vectors, their distribution in the landscape varied dramatically.

Co-infection of host plants by multiple viruses (as in the case of *T. repens* by AMV, ClYVV, and WClMV) may result in a reduction of the risks associated with the release of virus-specific resistant host genotypes. Alves-Júnior et al. (2009) demonstrated that symptoms were equivalent in *Nicotiana benthamiana* plants co-infected with tomato rugose mosaic virus (ToRMV) and tomato yellow spot virus (ToYSV) to those generated by ToYSV alone. Therefore, any competitive advantage associated with resistance to one virus may be reduced as a result of compensatory effects of infection by other viruses. Consequently the potential risks associated with release of nontarget white clover populations from the effects of an individual virus, i.e., AMV, ClYVV, or WClMV, may be reduced. Risk assessments need to consider not only the effects of the specific virus, to

which the plant is resistant, but also the possible compensatory effects of other pathogens present in the environment, particularly in a cases such as this where a large proportion of the wild plants were found to be co-infected with multiple viruses.

Finally, it is important to consider the relationship of nontarget plant communities with agricultural lands where disease resistant plants are likely to be grown. We found that those communities close to agricultural lands, with an abundant host population (often in regions where the host is grown commercially) or in disturbed areas (commonly due to agricultural activities) are more likely to be at risk following the release of AMV-resistant *T. repens*. If GM AMV-resistant *T. repens* is released commercially then these high risk sites are likely to be the ones closest to the site of commercial release and therefore the most difficult to protect. A detailed knowledge of host–pathogen spatial distribution in the potential release area is a crucial component of any environmental risk assessment of disease resistant plants.

Our results demonstrate the spatial and compositional complexity that can exist in widespread, natural multi-disease pathosystems: the factors influencing host–pathogen and pathogen–pathogen coexistence were numerous in this system. The process of a tiered risk assessment for PR species, in the absence of general principles that relate pathosystem structure and distribution and risk to nontarget habitats may lack rigor. While expensive and time-consuming to conduct, it is clear that if risk assessments of PR plants are to be effective they must account for variation in the target pathosystem.

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Supplemental Material

Appendix A

Key vegetation communities and habitats surveyed in the study region (*Ecological Archives* A022-002-A1).

Appendix B

Geographic location of sites with alfalfa mosaic virus, white clover mosaic virus, and clover yellow vein virus in the study region (*Ecological Archives* A022-002-A2).

Appendix C

Potential plant hosts for alfalfa mosaic virus present in New South Wales, Australia (*Ecological Archives* A022-002-A3).