Can sibling species explain the broadening of the host range of the broom seed beetle, *Bruchidius villosus* (F.) (Coleoptera: Chrysomelidae) in New Zealand?

Melanie L. Haines[1], Jean-François Martin[2,3], Rowan M. Emberson[1], Pauline Syrett[4], Toni M. Withers[5] and Sue P. Worner[1]

[1]Bio-Protection and Ecology Division, P.O. Box 84, Lincoln University, Canterbury, New Zealand. E-mail: melanielhaines@gmail.com

[2] CSIRO European Laboratory, Campus International de Baillarguet, 34980 Montferrier sur-Lez, France

[3] Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet, CS 30016, 34988 Montferrier-sur-Lez cedex, France

[4] Landcare Research, PO Box 69, Lincoln, New Zealand

[5] Forest Biosecurity and Protection, Ensis – a joint venture with CSIRO, Te Papa Tipu Innovation Park, 49 Sala Street, PB 3020, Rotorua

Abstract

Following introduction into New Zealand for biological control of Scotch broom, *Cytisus scoparius*, the broom seed beetle, *Bruchidius villosus*, was found utilising tagasaste, *Chamaecytisus palmensis*, which was not predicted by host range testing. Historical and more recent records of the host range of *B. villosus* are inconsistent. One possible explanation for these inconsistencies is that more than one species is included within the current concept of *B. villosus*. Sequence data from the mitochrondrial gene COI was used to determine whether there was genetic support for *B. villosus* as a single genetic entity with a broad host range within the Genisteae, or whether the nominal species is composed of two or more sibling species each with narrower host ranges. Samples of *B. villosus* collected from eight countries and five plant species were analysed. Results showed a low level of sequence polymorphism (0.8%) between individuals of *B. villosus* compared with the divergence between *B. villosus* and its congeners *B. lividimanus* and *B. seminarius* (8 and 12% respectively). This suggests that *B. villosus* is a single species with a broader host range than was predicted by host range tests.

Keywords: *Cytisus scoparius*, *Chamaecytisus palmensis*, Genisteae, mitochondrial DNA (COI)

Introduction

Bruchidius villosus (Fabricius) (Coleoptera: Chrysomelidae: Bruchinae), commonly known as the broom seed beetle, is native to Europe and has been introduced for biological control of Scotch broom, *Cytisus scoparius* (L.) Link into North America, New Zealand and Australia. In 1985, when *B. villosus* was identified as a potentially useful biological control agent against this widespread and damaging weed, host specificity testing was undertaken both in the UK and in quarantine in New Zealand. These tests indicated that it was host specific to *Cytisus* spp. and as a result, *B. villosus* (sourced from Silwood Park, UK) was released into New Zealand in 1986 (Syrett & O'Donnell 1987). Additional testing in Australia in 1994 led to the same conclusion and *B. villosus* was released there in 1995 (Hosking *et al*. 1996). However, in 1999 *B. villosus* was found emerging from seeds of *Chamaecytisus palmensis* (Christ) Bisby & K. Nicholls in the field at Lincoln, Canterbury (Syrett 1999). Recent field records from data collected in New Zealand and Europe have shown that beetles identified as *B. villosus* are utilising additional plant species in the Fabaceae, tribe Genisteae (Haines 2004, Sheppard *et al*. 2006).

Confusion surrounds the host range of *B. villosus* as there are many inconsistencies in the historical rearing and oviposition records. Some studies report that the host range of this insect includes many different pod-forming plants throughout the Genisteae (Zacher 1952a, Zacher

1952b, Frick 1962, Bottimer 1968, Szentesi & Wink 1991, Hosking 1992, Syrett & Emberson 1997, Syrett, 1999), while others suggest that it is more or less host specific to Scotch broom (Parnell 1966, Waloff 1968, O'Donnell & Manfield 1986, Syrett & O'Donnell 1987, Isaacson & Markin 1998).

There has also been uncertainty regarding the identity of *B. villosus*, which has also been referred to as *Bruchidius ater* (Marsham) (Parnell 1964, Parnell 1966, Bottimer 1968, Syrett & O'Donnell 1987), *Bruchidius fasciatus* (Oliver) (Hoffmann 1945, Frick 1962), *Sparteus villosus* (Fabricus) (Frick 1962), *Bruchidius cisti* (Paykel) (Zacher 1952a) and *B. villosus* (Fabricus) (Zacher 1952a, Zacher 1952b). All of these names are now generally accepted as referring to the species currently known as *B. villosus* (Southgate 1963, Aldridge & Pope 1986).

Field observations and initial data from 1999 (M. Haines unpubl.) and 2000 (Wittenberg & Thomann 2001) have suggested there is phenotypic plasticity in body size and colour of *B. villosus* depending on the host plant seeds in which they develop. Adults emerging from seeds of *Spartium junceum* (L.) and *Ch. palmensis* may be larger, and sometimes more gold or brown in colour, than those emerging from *C. scoparius*, which are smaller and darker. This could indicate that more than one species is included within *B. villosus*, and that sibling species were introduced into New Zealand, not all of which were represented in the population used for host testing. This has happened on a previous occasion when a second seedfly species, *Botanophila jacobaeae* (Hardy), was included in the population of the weed biological control agent, *B. seneciella* (Meade), introduced to New Zealand in 1936 for biological control of ragwort, *Senecio jacobaea* (L.) (Holloway 1983).

A species level investigation was carried out to determine whether the non-target impact in New Zealand is the result of the accidental introduction of a second cryptic or sibling species which varies in specificity from the tested population of *B. villosus*. The mitochondrial DNA gene region cytochrome oxidase subunit 1 (COI) was chosen for this characterisation as it has been a useful marker in previous studies of interspecies variation (reviewed in Caterino *et al*. 2000).

Materials and Methods

Insect material

Broom seed beetles were collected from different locations and host plants to detect the widest possible sequence variation and to enable assessment of genetic variability associated with host plant and geographic range (Table 1). Beetles were identified as *B. villosus* based on morphology (Aldridge & Pope 1986). Specimens of the related species *B. seminarius* (F.) and *B. lividimanus* (F.), both recognised as distinct species, were also sequenced. Finally, sequences of two other unrelated chrysomelid species were extracted from GenBank and used as outgroups to polarize substitutions along the phylogenetic tree of the *Bruchidius* genetic entities (see Table 1 for details).

Laboratory procedures

For the extraction of total DNA, a modified CTAB (hexadecyltrimethylammonium bromide) (Doyle & Doyle 1987) procedure was used. The individuals were homogenized in 500 μl of buffer, containing Tris 100 mM, pH 8.0, EDTA 20 mM, NaCl 0.7 M, CTAB 2%, PVP 360 w/v 1% and 2-mercaptoethanol 0.2%. For digestion, 10 μl Proteinase K (10 mg/μl) was added and the samples were kept in a water bath at 37°C for one hour. The samples were then purified using chloroform-isoamylic alcohol protein elimination, followed by a series of phenol-chloroform and chloroform wash steps. DNA was precipitated by adding 1/10 volume of NaAc 3 M and three volumes of cold ethanol (−20°C). The samples were then kept at −20°C for about one hour, followed by a centrifugation at 8,000 g for 15 min. The DNA was subsequently vacuum-dried and resuspended in ultra pure water.

For the PCR reaction, a mix of 2 μl DNA extract, 0.5 μM of each primer, 0.4 μM dNTP, 1 unit of Qiagen *Taq* polymerase and 1 X *Taq* buffer was prepared. PCR amplifications were done with a Hybaid PCR Express thermal cycler. Cycling parameters were: initial denaturation of 2 min and 5 cycles of 94°C denaturation (1 min), 48°C annealing (1 min), 62°C extension (1 min), followed by 35 cycles of 94°C denaturation (1 min), 52°C annealing (1 min), 62°C extension (1 min) with a final 62°C extension of 5 min. Primers used for COI amplification were C1-J-2183 and TL2-N-3014 as defined in Simon *et al*. (1994).

Table 1: Collections of *Bruchidius* used for molecular characterisation (COI) in this investigation.

No.	Taxon	Host Plant	Country	Date	Genbank Accession
1	*B. villosus*	*Laburnum anagyroides*	Hungary	08/01	DQ351960
2	*B. villosus*	*Genista monspessulana*	Spain	06/01	DQ351969
3	*B. villosus*	*G. monspessulana*	Spain	06/01	DQ351967
4	*B. villosus*	*Chamaecytisus palmensis*	New Zealand	12/01	DQ351959
5	*B. villosus*	*Cytisus scoparius*	New Zealand	11/01	DQ351962
6	*B. villosus*	*C. scoparius*	Germany	06/01	DQ351963
7	*B. villosus*	*C. scoparius*	England	08/01	DQ351965
8	*B. villosus*	*C. scoparius*	France	07/01	DQ351968
9	*B. villosus*	*C. scoparius*	France	07/01	DQ351964
10	*B. villosus* (black)	*Spartium junceum*	France	07/01	DQ351958
11	*B. villosus* (gold)	*S. junceum*	France	07/01	DQ351970
12	*B. villosus*	*C. scoparius*	Canada (B.C.)	08/03	DQ351966
13	*B. villosus*	*C. scoparius*	USA (Oregon)	08/03	DQ351961
1	*B. lividimanus*	*G. monspessulana*	Spain	06/01	DQ351971
2	*B. lividimanus*	*G. monspessulana*	Spain	06/01	DQ351972
1	*B. seminarius*	**Coronilla glauca*	France	07/01	DQ351973
2	B. seminarius	**Co. emerus*	France	07/01	DQ351974
	Diabrotica longicornis	–	–	–	AF278547
	Acalymma vittatum	–	–	–	AF278542

* = tribe Loteae. All other host plants Genisteae

From each population under investigation, one or two individuals were randomly chosen and from each approximately 800 bp of COI were amplified using the above described primers. The amplified segments were sequenced using the XL capillary automated sequencer Big Dye Terminator ABI 3.1 chemistry and analysed in an automated capillary sequencer (ABI 3130 XL). Each PCR product was sequenced in both directions and the same PCR primers were used in the sequencing reaction.

Data analysis

The sequences obtained were first checked by eye then aligned using the software ClustalX (Thompson *et al*. 1997). The sequences were then individually examined for protein coding frame-shifts to ensure the absence of pseudogenes (Zhang & Hewitt 1996) and aligned. Mega 2.1 (Kumar *et al*. 1993) was used to estimate all the statistics relative to the sequences (base composition, nucleotide diversity, distance within and between groups and coefficient of differentiation). The neighbour-joining clustering algorithm was applied (Saitou & Nei 1987) on a Jukes-Cantor distance to account for multiple substitutions. The bootstrap test (1000 replicates) was used to evaluate the reliability of the topology obtained (Felsenstein 1985).

Results

A total of 475 base pairs was available for all taxa and provided 119 variable sites in the data set, 91 of which were parsimony-informative. Initially 800 bases were amplified but the quality of some sequences was poor resulting in the removal of part of the 5’ and 3’ sides from the total fragment. The size of the alignable sequences with other chrysomelids was another constraint, which led to a 475 base pair reliable alignment. No bias in base composition was observed (chi-square test with $\alpha = 5\%$ $P = 0.014$), while all sequences were A-T rich, which is typical in insects (Lunt *et al*. 1996). The distance, based on percentage of divergence between pairs of sequences computed among

putative *B. villosus* individuals ranged from zero (100% identity between individuals collected from the following pairs of host plants: *G. monspessulana* (Spain) and *C. scoparius* (France), *L. anagyroides* (Hungary) and *C. scoparius* (USA), and *C. scoparius* (England and Canada)), to 0.0075 (three mutations) with nucleotide diversity (Nei, 1987) $\pi = 0.004 \pm 0.002$. These data are compatible with intra-specific variation in Coleoptera and other insects (Simon *et al*. 1994, Hebert *et al*. 2003). Further, calculation of the mean percentage of divergence between specimens of *B. villosus* and representatives of *B. lividimanus* ($D = 0.083 \pm 0.013$) and *B. seminarius* ($D = 0.134 \pm 0.017$) clearly indicate differentiation of the three recognized entities based on this mtDNA marker (coefficient of differentiation $D = 0.972 \pm 0.011$). The divergences between these three entities are typical of inter-specific level differentiation (Hebert *et al*. 2003).

The tree reconstruction based on Neighbour-joining shows that *B. villosus* is a monophyletic clade strongly supported by bootstrap proportions (BP = 100), with *B. lividimanus* falling as a sister group (BP = 99) (Fig. 1). The tree was rooted using sequences of two other chrysomelids (*Diabrotica longicornis* (Say) and *Acalymma vittatum* (F.)). Within *B. villosus* there is no significant trend to cluster sequences according to host plants or geographic locations, except perhaps for specimens found on *S. junceum* that cluster together with some support (BP=68) (Fig. 1, No. *10* and *11*).

Discussion

The observed differentiation between the sequences of individuals recognised as *B. villosus* is fully compatible with levels observed at the intraspecific level in Simon *et al*. (1994) and Hebert *et al*. (2003). The relationships between the three species of *Bruchidius* used in this study agree with those found by Kergoat *et al*. (2004) in a wider phylogenetic analysis of the genus. *Bruchidius lividimanus* is the sister species of all *B. villosus* specimens sequenced and *B. seminarius* is more distantly related to this species pair. Based on these sequence data, there is no strong evidence to suggest that *B. villosus* reared from different host plants comprised more than one species. However, the morph found on *S. junceum* appears as a separate cluster among other *B. villosus* samples and could indicate current phenological separation of the host plant population represented by these individuals, although very few characters (mutations) support this grouping as the selected marker focuses on species level information. To see whether host races of beetles or biotypes amongst plants might explain why the host range of *B. villosus* is broader than predicted by host specificity tests, more specimens and more variable genes, or DNA fingerprinting techniques such as AFLD (Vos *et al*. 1995), could be used.

Developmental polyphenisms, such as the size and colour variation in the populations of *B. villosus* reared from *S. junceum*, can occur when phenotypic variation (or plasticity) is produced by differences in environmental conditions rather than by differences in genetic constitution. For example differences in colour forms of *Nemoria arizonaria* (Grote) (Geometridae) caterpillars occur as a result of larval diet alone (Greene 1996, Greene 1999). Diet also alters horn-length/body size allometry in the beetles *Onthophaus acuminatus* Harold (Scarabaeidae) (Emlen 1994), and, particularly relevant to the present study, differences in body weight of the seed beetle *Stator limbatus* (Horn) (Bruchidae) are affected by the size of the seed from which individuals emerge (larger seeds produce beetles larger than those developing in smaller seeds) (Fox 1997). Larval diet may therefore explain the differences in phenotype observed between individuals of *B. villosus* reared from different host seeds.

Although the number of specimens used in this study was limited, and only a single gene region was investigated, these results indicate that *B. villosus* is a single species with a broader host range than was predicted by host range testing.

Acknowledgements

The authors are grateful for funding for this research from the New Zealand Vice-Chancellors Committee, the Miss E. L. Hellaby Indigenous Grassland Trust, Lincoln University, CSIRO Entomology Division and the Foundation for Research, Science and Technology Contract No. C09X0210. Arpad Szentesi, Ruediger Wittenberg, Thierry Thomann, Sylvie Agret, Simon Fowler, Dennis Isaacson and Judy Myers provided

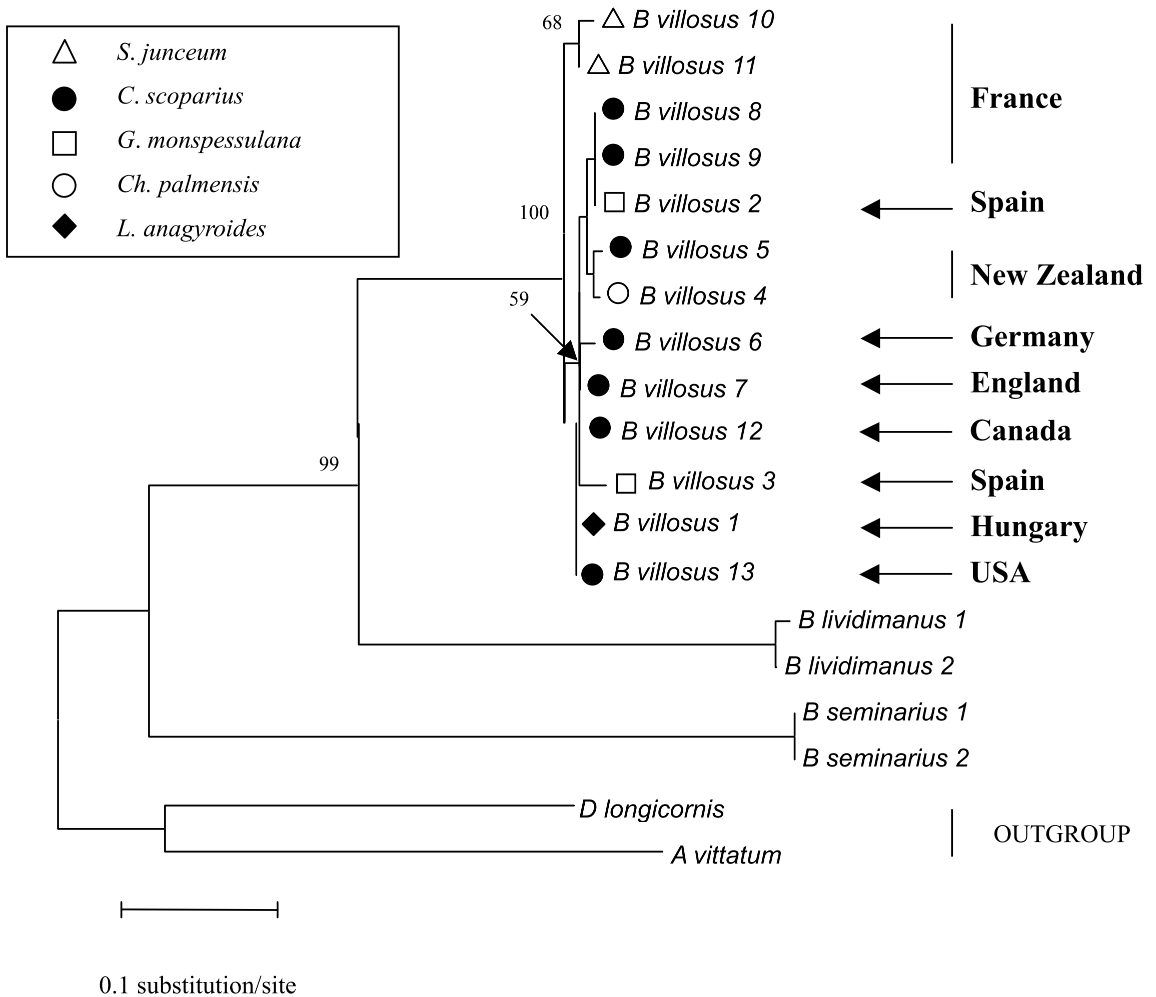

Figure 1. Neighbour-joining phylogram, where number of changes includes all nucleotide mutations. Numbers following species names refer to collections listed in Table 1.

invaluable field assistance and logistical support. Chris Frampton, Andy Sheppard, Warren Chinn, Cor Vink and two anonymous referees have all provided helpful input into this manuscript. We wish to thank all colleagues at the USDA and CSIRO European Laboratories for assisting with this project.

References

Aldridge RJW, Pope RD. 1986. The British species of *Bruchidius* Skilsky (Coleoptera: Bruchidae). *Entomologist's Gazette 37:* 181-193.

Bottimer LJ. 1968. On the two species of *Bruchidius* (Coleoptera: Bruchidae) established in North America. *The Canadian Entomologist 100:* 139-145.

Caterino MS, Cho S, Spelling FAH. 2000. The current state of insect molecular systematics: a thriving tower of Babel. *Annual Review of Entomology 45:* 1-54.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin 19:* 11-15.

Emlen D. 1994. Artificial selection on horn length-body size allometry in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Evolution 50:* 1219-1230.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution 39:* 783-791.

Fox C. 1997. The ecology of body size in a seed beetle, *Stator limbatus*: persistence of environmental variation across generations? *Evolution 51:* 1005-1010.

Frick KE. 1962. A three-week study of *Bruchidius villosus*, the seed beetle that attacks the seeds of *Cytisus scoparius* in the Eastern United States. USDA**.** pp. 10

Greene E. 1996. Effect of light quality and larval diet on morph induction in the polymorphic caterpillar *Nemoria arizonaria* (Lepidoptera: Geometridae). *Biological Journal of the Linnean Society 58:* 277-285.

Greene E. 1999. Phenotypic variation in larval development and evolution: polymorphism, polyphenism, and developmental reaction norms. In: *The origin and evolution of larval forms*. (eds B Hall, M Wake) pp. 379-410. Academic Press, San Diego.

Haines ML. 2004. The unpredicted host use of *Chamaecytisus prolifer* by the introduced weed biocontrol agent, *Bruchidius villosus*: a retrospective analysis and explanation. PhD Thesis, Lincoln University, Canterbury, New Zealand.

Hebert PDN, Ratnasingham S, de Waard JR. 2003. Barcoding animal life: cytochrome oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B 270:* 596-599.

Hoffmann A. 1945. Coléoptères Bruchides et Anthribides. *Faune de France 44:* 1-184.

Holloway BA. 1983. Species of ragwort seedflies imported into New Zealand (Diptera: Anthomyiidae). New Zealand Journal of Agricultural Research *26:* 245-249.

Hosking JR, Smith J, Sheppard A. 1996. Biology of Australian Weeds: *Cytisus scoparius* L., Scotch Broom. *Plant Protection Quarterly 11:* 102-108.

Hosking JR. 1992. The impact of seed and pod feeding insects on *Cytisus scoparius* (L.) Link. In: *Proceedings of the 8th International Symposium on Biological Control of Weeds* (eds ES Delfosse, RR Scott) pp. 1-13. Lincoln University, Canterbury, New Zealand.

Isaacson DL, Markin G. 1998. *A petition to import Bruchidius villosus from North Carolina to the Western U.S. for biological control of Scotch broom (Cytisus scoparius).* Oregon Department of Agriculture. Oregon. pp. 25.

Kergoat GJ, Delobel A, Silvain JF. 2004. Phylogeny and host-specificity of European seed beetles (Coleoptera: Bruchidae), new insights from molecular and ecological data. *Molecular Phylogenetics and Evolution 32:* 855-865.

Kumar S, Tamura K, Nei M. 1993. *A Guide to Molecular Evolutionary Genetics Analysis Program for Microcomputers Institute of Molecular Evolutionary Genetics.* Pennsylvania State University, University Park. Pennsylvania. pp. 140.

Lunt D, Zhang D, Syzymura J, Hewitt G. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Molecular Biology 5:* 153-165.

Nei M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York. pp. 512.

O'Donnell DJ, Manfield I. 1986. *Host specificity of Bruchidius ater (Marsham) (Coleoptera: Bruchidae) and Apion fuscirostre F. (Coleoptera: Curculionidae). Results of tests carried out in the U.K.* CAB International Institute of Biological Control. Ascot, U.K.

Parnell JR. 1964. The external morphology of the larvae and notes on the pupae of *Bruchidius ater* (Marsh.) (Col., Bruchidae) and *Apion fuscirostre* F. (Col., Curculionidae). *Entomologist's Monthly Magazine 100:* 83-87.

Parnell JR. 1966. Observations on the population fluctuations and life histories of the beetles *Bruchidius ater* (Bruchidae) and *Apion fuscirostre* (Curculionidae) on broom (*Sarothamnus scoparius*). *Journal of Animal Ecology 35:* 157-188.

Saitou N, Nei M. 1987. The neighbour-joining method – a new method for reconstructing

phylogenetic trees. *Molecular Biology and Evolution 4:* 406-425.

Sheppard A, Haines M, Thomann T. 2006. Native-range research assists risk analysis for non-targets in weed biological control: the cautionary tale of the broom seed beetle. *Australian Journal of Entomology 45:* 292-297.

Simon CF, Frati A, Beckenbach B, Crespi H, Lui, Flook P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and compilation of conserved PCR primers. *Annals of Entomological Society America 87:* 651-701.

Southgate BJ. 1963. The true identity of the broom bruchid (Coleoptera) and synonymic notes on other species of *Bruchidius*. *Annals of the Entomological Society of America 56:* 795-798.

Syrett P. 1999. Broom beetle gets taste for tagasaste. *Biocontrol News and Information 20:* 51.

Syrett P, Emberson RM. 1997. The natural host range of beetle species feeding on broom, *Cytisus scoparius* (L.) Link (Fabaceae), in Southwest Europe. *Biocontrol Science and Technology 7:* 309-326.

Syrett P, O'Donnell DJ. 1987. A seed-feeding beetle for biological control of broom. *Proceedings of the New Zealand Weed and Pest Control Conference 40:* 19-22.

Szentesi A, Wink M. 1991. Fate of quinolizidine alkaloids through three trophic levels: *Laburnum anagyroides* (Leguminosae) and associated organisms. *Journal of Chemical Ecology 17:* 1557-1573.

Thompson JD, Gibson TJ, Plenwinak F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research 24:* 4876-4882.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research 23(21):* 4407-4414.

Waloff N. 1968. Studies on the insect fauna of Scotch broom *Sarothamnus scoparius* (L.). *Advances in Ecological Research 5:* 87-208.

Wittenberg R, Thomann T. 2001. *Investigations on potential biological control agents of Scotch broom, Cytisus scoparius*. CABI Bioscience Switzerland Centre. Delemont, Switzerland**.** pp. 30

Zacher F. 1952a. Die Nahrpflanzen der Samenkafer. *Zeitschrift fur angewandte Entomologie 33:* 210-217.

Zacher F. 1952b. Die Nahrpflanzen der Samenkafer. *Zeitschrift fur angewandte Entomologie 33:* 460-480.

Zhang D, Hewitt G. 1996. Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution 11:* 247-251.