Heredity **75** (1995) 243–255

Received 28 November 1994

Genetic continuity within, and discontinuities among, populations of leafroller moths with distinct sex-pheromones

CHRISTOPHER S. WHITE & DAVID M. LAMBERT*

Ecology and Evolution, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

Species belonging to two genera of leafroller moths have been shown to exhibit considerable variation in sex-pheromones. Six different pheromone types have been described, three each belonging to *Planotortrix excessana* and *Ctenopseustis obliquana*. We report here the existence of homozygous genotypes in wild-caught individuals and that no heterozygous genotypes for any of the presumptive genetic loci were recorded, despite electrophoresis of 820 individuals belonging to two pheromone types of *P. excessana* and 781 moths belonging to the two pheromone types of *C. obliquana*. This is particularly significant given that more than 500 individuals of each pair were collected from areas of sympatry. Hence these data unequivocally support the specific status of *P. excessana* pheromone type A and type B&C and *C. obliquana* type I&III and type II. A further study of 30 presumptive genetic loci of these pheromonally-distinct cryptic species reveals considerable differences in allele frequencies at other loci and shows that within pheromonal types there is a remarkable stability in gene frequencies, with little clinal variation. The variation within pheromone types that does exist results largely from regionally-distinct populations which are either geographically isolated, or subject to a range of human disturbances. The bulk of the total genetic variation within the taxonomically-described entities is among the pheromonally-distinct species. The question of the specific status of two pheromonally-distinct but allopatric populations is addressed, and it is suggested that genetic data cannot unequivocally resolve the specific status of such groups.

Keywords: cryptic species, *Ctenopseustis obliquana*, isozymes, leafroller moths, pheromone strains, *Planotortrix excessana*.

Introduction

The biological status of New Zealand leafroller pheromone types within the existing (Dugdale, 1966) classifications *Planotortrix excessana* and *Ctenopseustis obliquana* has been under examination for several years (Foster *et al.*, 1986, 1989, 1991; Foster & Roelofs, 1987; Dugdale, 1990). These studies have resulted in the suggestion that the pheromone types represent distinct species on the following bases.

(1) The pheromone types possess different long-range chemical signalling systems used in reproduction (Foster *et al.*, 1986, 1989; Foster & Roelofs, 1987).
(2) The females of each type attract only males of the same type in mating trials (Foster *et al.*, 1991).
(3) Some minor morphological differences have been discovered that can at least partially distinguish the types (Dugdale, 1990).
(4) Electrophoretic markers distinctive to the laboratory-reared lines of different pheromonal types have been described (Foster *et al.*, 1991).

The above criteria have resolved two main types within each taxonomically described species; *P. excessana* type A and type B&C, and *C. obliquana* type I&III and type II. All of the above criteria coincide for these groupings with two exceptions. In the North Island (Fig. 1), *C. obliquana* type II cannot be separated from type I&III on the basis of the morphological distinction, but remains consistent with type II for genetic markers, pheromone signals and mating trials (Foster *et al.*, 1990). The second exception is for *P. excessana* types found on the Chatham Islands. These have been designated *P. octoides* by Dugdale

*Correspondence.

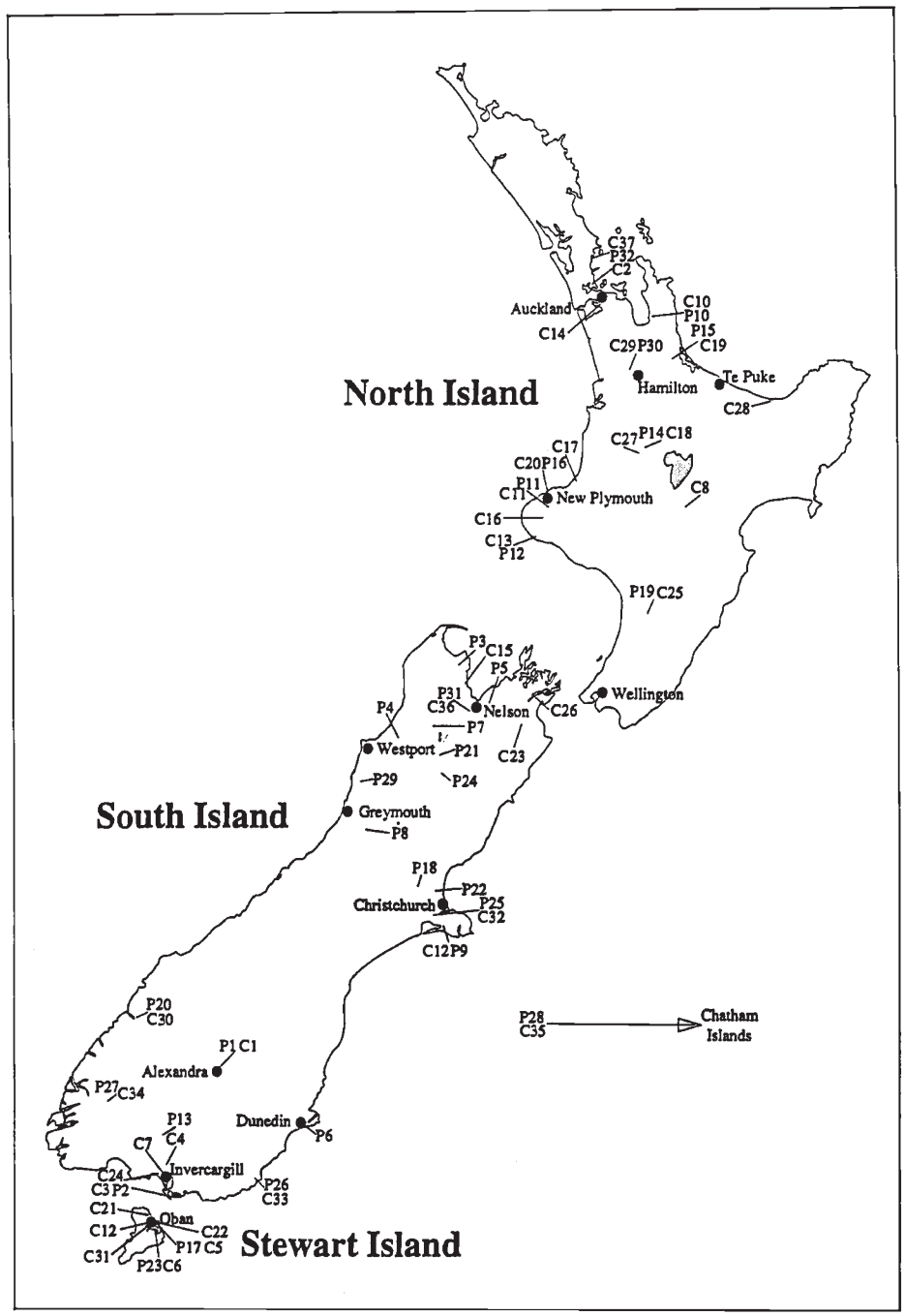

Fig. 1 Locations where *Planotortrix excessana* and *Ctenopseustis obliquana* pheromone types were sampled in this study.

(1990) on the basis of morphological characters — although they have type A pheromone signalling systems. No mating trials have been conducted between *P. octoides* and mainland type A individuals.

Differences in the specific-mate recognition systems (SMRSs) (Paterson, 1985; Lambert *et al.*, 1987; McEvey, 1992; Lambert & Spencer, 1995) of pheromone types are of critical importance in determining their status as biological species. Thus, the pheromone signalling differences, which originally drew attention to the possibility of cryptic species within the original taxa (Galbreath *et al.*, 1985), still provide the strongest evidence of new species. These differences are likely to be of importance given the mating and response trials conducted both in field cages and wind tunnels (Foster *et al.*, 1991).

Despite the body of evidence available, it is difficult to confirm that the pheromone types represent species

with unique SMRSs in their typical environment. This could be directly tested by observing the outcome of matings in sympatric populations of the respective pheromone types. If these matings defined groups concordant with the pheromone types, their specific status would be supported. Of course markers can be used to recognize genetic discontinuities which may result from the existence of a number of species. Such an approach, while providing a great deal of information on the genetic structuring of natural populations is dependent upon finding a consistent genetic marker for each type. In the case of these leafroller moths, the β-hydroxybutyrate dehydrogenase-3 locus has been shown to discriminate between some laboratory-reared strains of the two *P. excessana* types and the hexokinase-1 locus similarly distinguishes between some strains of the *C. obliquana* types (Foster *et al.*, 1991; White & Lambert, 1994). These electrophoretic differences were also shown, at least for the *C. obliquana* types, to produce an identifiable marker for heterozygotes between them in forced laboratory crosses. These electrophoretic markers can then be used to examine the genetic outcome of reproduction in natural populations as they may indicate the species to which any collected individuals belong.

This study aimed to investigate the genetic structure of wild populations of these leafrollers belonging to these two genera. In particular the following specific questions were of interest.

(1) Would electrophoretic markers which have been shown to discriminate among laboratory-reared strains of leafroller moths also discriminate among wild-caught individuals belonging to different pheromonal types, in that no heterozygous genotypes would be detected?
(2) Would the wider genetic structuring of the wild populations support the groupings recognized from laboratory-reared populations?
(3) Would the geographical distribution of electrophoretically-identified groupings match those of pheromonally- and morphologically-identified groupings?

The confirmation of these points would establish that the pheromone differences in SMRSs among the types result in cohesive reproductive communities in the natural habitat. In addition, the genetic structuring of populations across geographical regions and between individual sample sites can be examined to reveal the reproductive dynamics within each type and assist in the resolution of residual questions over the status of North Island *C. obliquana* type II and *P. octoides*. For the purposes of all the analyses, North Island type II are treated as part of the rest of type II and *P. octoides* is treated as *P. excessana* type A.

Materials and methods

Specimens examined

Samples of *P. excessana* and *C. obliquana* were collected from throughout mainland New Zealand and from the Chatham Islands. The sampling sites for *P. excessana* and *C. obliquana* are shown on Fig. 1 and the locations of each listed with the accompanying code in Table 1. Individuals were collected as either eggs, larvae, pupae or adults and, whenever possible, kept alive until transported to the laboratory. Upon arrival adults were frozen immediately at $-80°C$, while earlier stages were kept in culture until they reached the adult form and were then frozen in the same manner.

Electrophoresis procedures

In all analyses 'Cellogel' electrophoresis medium was used and buffers and strains were as detailed by Richardson *et al.* (1986). First, all samples were analysed electrophoretically for either the β-hydroxybutyrate dehydrogenase or the hexokinase enzyme systems, depending on the taxonomic grouping to which they belonged. Secondly, individuals from sampling sites that produced near to, or more than 20

Table 1 Sample sites, numbers of moths and numbers of each pheromone type of *Planotortrix excessana* and *Ctenopsensis obliquana* recorded in this study

Sample code	Sample site	Number analysed	Number of A	Number of B&C
P1	Alexandra	62	28	34
P2	Bluff Hill	40	18	22
P3	Caanan	49	19	30
P4	Dublin Tce	3	0	3
P5	Dun Mountain	12	7	5
P6	Dunedin	2	1	1
P7	Hope River	4	3	1
P8	Jacksons	7	5	2
P9	Kaituna	4	0	4
P10	Kauranga Valley	4	4	0
P11	Lake Mangamahoe	1	0	1
P12	Manaia	5	2	3
P13	Moss Bush	5	1	4
P14	Mt Pureora	7	3	4
P15	Mt Te Aroha	9	2	7
P16	New Plymouth	14	4	10
P17	Oban	97	0	97
P18	Okuku	8	0	8
P19	Palmerston North	56	33	23
P20	Sandfly Point	3	2	1
P21	Shenandoah	38	18	20

Table 1 *Continued*

Sample code	Sample site	Number analysed	Number of A	Number of B&C
P22	St Albans	3	2	1
P23	Stewart Island	3	0	3
P24	Sylvia Flats	8	2	6
P25	Tai Tapu	49	29	20
P26	Tautuku	6	2	4
P27	Te Anua	11	0	11
P28	Te Matarae	98	98	0
P29	Truman Track	54	20	34
P30	Waikato	117	73	44
P31	Wairoa Gorge	38	17	21
P32	Wenderholm	3	3	0

Sample code	Sample size	Number analysed	Number of I&III	Number of II
C1	Alexandra	11	11	0
C2	Birkenhead	4	4	0
C3	Bluff Hill	49	28	21
C4	Forest Hill	8	5	3
C5	Fred's Camp	5	4	1
C6	Horseshoe Bay	42	25	17
C7	Invercargill	2	2	0
C8	Kaimanawas	26	24	2
C9	Kaipipi Creek	1	1	0
C10	Kauranga Valley	12	11	1
C11	Lake Mangamahoe	12	9	3
C12	Little River	8	5	3
C13	Manaia	10	8	2
C14	Manurewa	22	19	3
C15	Mariri	2	2	0
C16	Mt Egmont	7	7	0
C17	Mt Messenger	1	1	0
C18	Mt Pureora	3	3	0
C19	Mt Te Aroha	4	4	0
C20	New Plymouth	9	5	4
C21	Oban	167	97	70
C22	Observation Rock	2	0	2
C23	Onamalutu	51	18	33
C24	Otatara	2	0	2
C25	Palmerston North	11	11	0
C26	Pelorus Sound	2	1	1
C27	Pureora	2	2	0
C28	Raroa	3	3	0
C29	Rukuhia	83	50	33
C30	Sandfly Point	3	1	2
C31	Stewart Island	51	23	28
C32	Tai Tapu	5	5	0
C33	Tautuku	7	5	2
C34	Te Anau	57	20	37
C35	Te Matarae	81	0	81
C36	Wairoa Gorge	13	5	8
C37	Wenderholm	18	18	0

Sample codes P1–P32 refer to *P. excessana*; codes C1–C37 refer to *C. obliquana*.

individuals of either type were analysed for all loci which were polymorphic in laboratory populations, i.e. a total of 26 polymorphic and four monomorphic loci. A maximum of 50 individuals of any one type were analysed from each site. The 22 enzyme systems screened were: aconitate hydratase (Acon; EC 4.2.1.3), adenosine deaminase (Ada; 3.5.4.4), adenylate kinase (Ak; 2.7.4.3), aldehyde oxidase (Ao; 1.2.3.1), esterase (Est; 3.1.1.1), fumarate hydratase (Fum; 4.2.1.2), aspartate aminotransferase (Got; 2.6.1.1), glucose-6-phosphate dehydrogenase (G6pd; 1.1.1.49), glycerol-3-phosphate dehydrogenase (αGpd; 1.1.1.8), glucose-phosphate isomerase (Gpi; 5.3.1.9), glutathione reductase (Gsr; 1.6.4.2), glucuronidase (Gus; 3.2.1.31), β-hydroxybutyrate dehydrogenase (Hbdh; 1.1.1.30), hexokinase (Hk; 2.7.1.1), isocitrate dehydrogenase (Idh; 1.1.1.42), malate dehydrogenase (Mdh; 1.1.1.37), malic enzyme (Me; 1.1.1.40), mannose phosphate isomerase (Mpi; 5.3.1.8), phosphoglycerate mutase (Pgam; 2.7.5.3), phosphoglucomutase (Pgm; 5.4.2.2), xanthine dehydrogenase (Xdh; 1.1.1.204) and xanthine oxidase (Xo; 1.2.3.2).

Results

To confirm the discrimination of the two loci for wild-caught individuals, a total of 820 moths of *P. excessana* were analysed for the *Hbdh-3* locus. Of these, 396 were homozygous for a fast electrophoretic variant and were therefore apparently of type A. The remaining 424 individuals were homozygous for a slower variant at the same locus and were consequently regarded as belonging to type B&C. The sample locations and the numbers analysed and their allocation to the two types are shown in Table 1 and Fig. 1. For the *C. obliquana* samples a total of 796 individuals were analysed for the *Hk-1* locus. Of this sample, 437 moths were homozygous for a slow electromorph, indicating they were of type I&III, and a further 359 individuals were homozygous for a faster variant and therefore were assigned to type II. The locations and numbers analysed for these samples are also shown in Table 1 and Fig. 1, along with the allocation into each type at each site. No heterozygous genotypes were recorded within any of the pheromone types, despite screening over 500 individuals for each pair of congeneric species from localities where the types were sympatric. In addition, all individuals that were successfully scored could be allocated to the four electrophoretic groups identified in laboratory populations, i.e. no new electromorphs for the marker loci were discovered in the wild-collected individuals.

The populations that had sufficient numbers for analysis for the remainder of the 22 enzyme systems listed earlier are shown in Table 2. The distributions of

Table 2 Locations and sample sizes of wild populations of leafroller moths which were analysed for 22 enzyme systems

Sample code	Sample site	Region	Taxonomic group	Pheromone type	Number analysed
C3	Bluff Hill	SSI	*C. obliquana*	I&III	28
				II	21
C6	Horseshoe Bay	STI	*C. obliquana*	I&III	25
				II	17
C8	Kaimanawas	NI	*C. obliquana*	I&III	24
C14	Manurewa	NI	*C. obliquana*	I&III	19
C21	Oban	STI	*C. obliquana*	I&III	50
				II	50
C23	Onamalutu	NSI	*C. obliquana*	I&III	18
				II	33
C29	Rukuhia	NI	*C. obliquana*	I&III	50
				II	33
C31	Stewart Island	STI	*C. obliquana*	I&III	23
				II	28
C34	Te Anau	SSI	*C. obliquana*	I&III	20
				II	37
C35	Te Matarae	CHI	*C. obliquana*	II	45
C37	Wenderholm	NI	*C. obliquana*	I&III	18
P1	Alexandra	SSI	*P. excessana*	A	28
				B&C	34
P2	Bluff Hill	SSI	*P. excessana*	A	18
				B&C	22
P3	Caanan	NSI	*P. excessana*	A	19
				B&C	30
P17	Oban	STI	*P. excessana*	B&C	50
P19	Palmerston North	NI	*P. excessana*	A	33
				B&C	23
P21	Shenandoah	NSI	*P. excessana*	A	18
				B&C	20
P25	Tai Tapu	NSI	*P. excessana*	A	29
				B&C	20
P28	Te Matarae	CHI	*P. excessana*	A	36
P29	Truman Track	NSI	*P. excessana*	A	20
				B&C	34
P30	Waikato	NI	*P. excessana*	A	50
				B&C	44
P31	Wairoa Gorge	NSI	*P. excessana*	A	17
				B&C	21

NI, North Island; NSI, north of the South Island above and including Chirstchurch; SSI, south of theSouth Island below Christchurch; STI, Stewart Island; CHI, Chatham Islands.

P. excessana types were largely sympatric with two notable exceptions; P17 was type B&C only and P28 was type A only (Table 1).

The *C. obliquana* type II had a patchy distribution in the North Island but was found at all other sites except C1. Only type II was found at site C35. Type I&III was found in the North Island, South Island and Stewart Island.

Analyses of genetic equilibrium

The electromorph frequencies for all loci for all species are presented in Tables 3–6. Hardy–Weinberg analyses revealed a consistent pattern of no significant deviation from equilibrium for almost all the populations, including the Chatham Island population of *P. excessana* type A. The relatively small sample sizes of many of the

Table 3 Electromorph frequencies in wild populations of *Planotortrix excessana* type A

Locus	Electromorph	Sample codes									
		P1	P25	P2	P28	P3	P31	P19	P30	P21	P29
Acon-1	a	0.304	0.259	0.333	0.083	0.316	0.324	0.152	0.210	0.333	0.325
	b	0.446	0.483	0.417	0.875	0.447	0.441	0.515	0.550	0.444	0.425
	c	0.250	0.259	0.250	0.042	0.237	0.235	0.333	0.240	0.222	0.250
Ada	b	0.321	0.293	0.333	0.042	0.368	0.353	0.288	0.170*	0.333	0.325
	c	0.679	0.707	0.667	0.958	0.632	0.647	0.712	0.830*	0.667	0.675
Ak	a	0.143	0.190	0.194	—	0.211	0.235	0.152	0.290	0.222	0.200
	b	0.554	0.552	0.500	0.833	0.553	0.529	0.621	0.450	0.528	0.550
	c	0.304	0.259	0.306	0.167	0.237	0.235	0.227	0.260	0.250	0.250
Est-2	b	0.804	0.724	0.806	0.625	0.816	0.853	0.803	0.780	0.806	0.800
	c	0.196	0.276	0.194	0.375	0.184	0.147	0.197	0.220	0.194	0.200
Fum	a	0.036	—	0.028	—	0.053	0.059	—	—	—	0.025
	b	0.964	1.000	0.972	1.000	0.947	0.941	1.000	1.000	1.000	0.975
αGpd	a	0.911	0.879	0.861	1.000	0.868	0.853	0.924	0.980	0.806	0.875
	b	0.089	0.121	0.139	—	0.132	0.147	0.076	0.020	0.194	0.125
Gpi	b	0.036	—	0.056	0.222	0.053	0.029	0.015	—	0.056	0.075
	c	0.875	0.914	0.861	0.681	0.895	0.912	0.924	0.870	0.889	0.875
	d	0.089	0.086	0.083	0.097	0.053	0.059	0.061	0.130	0.056	0.050
Gus	c	0.214	0.190	0.250	0.472	0.237	0.265	0.091	0.120	0.250	0.250
	d	0.786	0.810	0.750	0.528	0.763	0.735	0.909	0.880	0.750	0.750
Hk-2	a	0.250	0.310	0.194	—	0.211	0.235	0.288	0.210	0.139	0.175
	b	0.750	0.690	0.806	1.000	0.789	0.765	0.712	0.790	0.861	0.825
Me	b	0.268	0.259	0.250	0.361	0.211	0.206	0.242	0.380	0.194	0.225
	c	0.732	0.741	0.750	0.639	0.789	0.794	0.758	0.620	0.806	0.775
Mpi	f	0.375	0.310	0.333	0.417	0.289	0.294	0.394	0.350	0.333	0.375
	g	0.446	0.448	0.472	0.583	0.500	0.500	0.500	0.540	0.444	0.400
	h	0.179	0.241	0.194	—	0.211	0.206	0.106	0.110	0.222	0.225
Pgam	a	0.036	0.034	—	—	—	—	0.076	—	0.028	0.050
	b	0.964	0.966	1.000	1.000	1.000	1.000	0.924	1.000	0.972	0.950
Pgm	a	0.589	0.586	0.528	0.375	0.632	0.647	0.606	0.510	0.667	0.675
	b	0.411	0.414	0.472	0.625	0.368	0.353	0.394	0.490	0.333	0.325

*Significant deviation from H.–W. (sample codes as in Table 2).
The following electromorphs were fixed: *Acon-2* a, *Ao* a, *Est-1* a, *Got-1* a, *Got-2* a, *G6pd* a, *Gsr* a, *Hbdh-1* a, *Hbdh-2* a, *Hbdh-3* b, *Hk-1* a, *Idh-1* a, *Idh-2* b, *Mdh-1* a, *Mdh-2* b, *Xdh* a, *Xo* a.

populations, combined with the presence of three electromorphs for several loci, made these tests relatively insensitive in many cases. However, even quite large samples, such as those from Stewart Island, showed no significant deviation from the expected equilibrium frequencies.

The exceptions to the above generality were in two sets of populations and the deviations from equilibrium are shown in the frequency Tables 3–6. The first set were populations that were not geographically widely separated from others, but found near areas of human activity. For *P. excessana* type A and both *C. obliquana* types, these populations showed deviation from Hardy–Weinberg at one or more loci. This was particularly notable for the *C. obliquana* types for which these populations were also divergent in every other genetic measure. The second set of populations are represented by the Chatham Island *C. obliquana* type II population which showed significant deviations from equilibrium at four loci. This was not paralleled by any similar deviation in the *P. excessana* type A population from the Chathams.

The source of the deviation from equilibrium was the same in every case. All deviating loci in the populations concerned showed an excess of homozygotes. However, in most cases the disequilibrium was also associated with a difference in the frequencies of the electromorphs for the locus in question, suggesting that the data reflected some kind of active process, rather than an epiphenomenon of the electromorph scoring technique.

Table 4 Electromorph frequencies in wild populations of *Planotortrix excessana* type B&C

Locus	Electromorph	Sample codes									
		P1	P25	P2	P3	P31	P19	P17	P30	P21	P29
Acon-1	a	0.162	0.100	0.159	0.167	0.190	0.217	0.190	0.261	0.225	0.235
	b	0.485	0.450	0.477	0.517	0.524	0.435	0.530	0.386	0.475	0.471
	c	0.353	0.450	0.364	0.317	0.286	0.348	0.280	0.352	0.300	0.294
Ada	b	0.574	0.550	0.591	0.600	0.571	0.500	0.630	0.545	0.675	0.647
	c	0.426	0.450	0.409	0.400	0.429	0.500	0.370	0.455	0.325	0.353
Ak	a	0.162	0.100	0.159	0.167	0.190	0.217	0.190	0.261*	0.225	0.250
	b	0.485	0.450	0.477	0.517	0.524	0.435	0.530	0.386*	0.700	0.691
	c	0.353	0.450	0.364	0.317	0.286	0.348	0.280	0.352*	0.050	0.059
αGpd	a	0.985	0.975	1.000	0.983	1.000	1.000	0.960	1.000	0.975	0.985
	b	0.015	0.025	—	0.017	—	—	0.040	—	0.025	0.015
Gpi	b	0.279	0.250	0.295	0.267	0.286	0.217	0.310	0.193	0.300	0.294
	c	0.721	0.750	0.705	0.733	0.714	0.783	0.690	0.807	0.700	0.706
Gus	c	0.853	0.825	0.909	0.917	0.905	0.804	0.880	0.795	0.925	0.926
	d	0.147	0.175	0.091	0.083	0.095	0.196	0.120	0.205	0.075	0.074
Hk-2	a	0.279	0.250	0.318	0.350	0.310	0.283	0.310	0.250	0.325	0.309
	b	0.721	0.750	0.682	0.650	0.690	0.717	0.690	0.750	0.675	0.691
Mdh-2	a	0.897	0.800	0.864	0.917	0.905	0.870	0.930	0.795	0.925	0.912
	b	0.103	0.200	0.136	0.083	0.095	0.130	0.070	0.205	0.075	0.088
Me	b	0.647	0.675	0.705	0.700	0.690	0.587	0.680	0.602	0.675	0.691
	c	0.353	0.325	0.295	0.300	0.310	0.413	0.320	0.398	0.325	0.309
Mpi	f	0.235	0.200	0.295	0.250	0.286	0.196	0.280	0.250	0.300	0.338
	g	0.765	0.800	0.705	0.750	0.714	0.804	0.720	0.750	0.700	0.662
Pgam	a	0.176	0.200	0.114	0.100	0.119	0.217	0.130	0.080	0.100	0.147
	b	0.824	0.800	0.886	0.900	0.881	0.783	0.870	0.920	0.900	0.853
Pgm	a	0.221	0.225	0.227	0.150	0.190	0.261	0.110	0.295	0.100	0.147
	b	0.779	0.775	0.773	0.850	0.810	0.739	0.890	0.705	0.900	0.853

*Significant deviation from H.–W. (sample codes as in Table 2).
The following electromorphs were fixed: *Acon-2* a, *Ao* a, *Est-1* a, *Est-2* b, *Fum* b, *Got-1* a, *Got-2* a, *G6pd* a, *Gsr* a, *Hbdh-1* a, *Hbdh-2* a, *Hbdh-3* a, *Hk-1* a, *Idh-1* a, *Idh-2* b, *Mdh-1* a, *Xdh* a, *Xo* a.

Genetic heterogeneity

To examine genetic structuring, hierarchical heterogeneity χ^2 analyses were performed for all cases where there was more than one population of any type within a region. The populations were divided into regions as shown in Table 7. There was no detailed biogeographical rationale for the regional divisions made, other than a correlation with the major spatial and geographical discontinuities between the groups of populations sampled. To examine heterogeneity amongst all populations of each type, heterogeneity analyses were also performed across all the regions. The results are shown only for the comparison of loci for which significant heterogeneity was recorded.

For the *P. excessana* no significant heterogeneity was recorded amongst the populations of either *P. excessana* type within the regions described (Table 7). The single exception was the *Pgam* locus for which there was a significant deviation for both types in the North Island region. Examination of the electromorph frequency data suggests this arose mainly from differences between the P30 and P19 populations at this locus.

For the analysis across all regions, the type A populations showed significant heterogeneity for 9 out of 13 loci (Table 8). From the frequency data for the most heterogeneous loci (*Gpi* and *Gus*) the main differences are found between the Chatham Island (P28) population and all other populations. The results for the type B&C populations tend to support this. No significant heterogeneity was observed at any locus for this type.

The results of the heterogeneity analyses for the *C. obliquana* showed no significant heterogeneity amongst the populations within the South Island and Stewart Island regions for either type I&III or type II. However, for the North Island region, the type I&III populations showed significant heterogeneity at 14 out of 16 loci, with 10 of these showing highly significant ($P<0.01$) heterogeneity (Table 7). This high level of

Table 5 Electromorph frequencies in wild populations of *Ctenopseustis obliquana* type I&III

Locus	Electromorph	Sample codes									
		C37	C14	C8	C3	C23	C6	C21	C31	C34	C29
Acon-1	a	0.079	0.056	0.104	0.089	0.111	0.080	0.110	0.043	0.075	0.030
	b	0.842	0.944	0.833	0.804	0.833	0.760	0.790	0.804	0.825	0.920
	c	0.079	—	0.063	0.107	0.056	0.160	0.100	0.152	0.100	0.050
Ada	a	0.632	0.417*	0.583	0.679	0.583	0.720	0.740	0.761	0.575	0.370*
	c	0.053	—	0.063	—	0.056	0.020	0.010	—	0.050	—
	d	0.316	0.583*	0.354	0.321	0.361	0.260	0.250	0.239	0.375	0.630*
Ao	a	0.553	0.333	0.583	0.625	0.583	0.660	0.610	0.696	0.625	0.790*
	b	0.447	0.667	0.417	0.375	0.417	0.340	0.390	0.304	0.375	0.210*
Est-2	a	0.684	0.889*	0.708	0.625	0.667	0.600	0.630	0.652	0.600	0.930
	b	0.316	0.111*	0.292	0.375	0.333	0.400	0.370	0.348	0.400	0.070
Fum	b	0.079	—	0.083	0.161	0.111	0.180	0.130	0.130	0.175	—
	c	0.921	1.000	0.917	0.839	0.889	0.820	0.870	0.870	0.825	1.000
α Gpd	b	0.026	—	0.021	0.089	0.056	0.080	0.100	0.109	0.075	—
	c	0.868	0.667*	0.875	0.804	0.750	0.800	0.790	0.783	0.825	0.960
	d	0.105	0.333*	0.104	0.107	0.194	0.120	0.110	0.109	0.100	0.040
Gpi	a	0.079	—	0.104	0.089	0.083	0.080	0.080	0.065	0.100	—
	b	0.842	1.000	0.771	0.750	0.833	0.740	0.780	0.826	0.775	1.000
	c	0.079	—	0.125	0.161	0.083	0.180	0.140	0.109	0.125	—
Gsr	a	0.289	0.083	0.250	0.214	0.250	0.300	0.310	0.283	0.300	0.400*
	b	0.711	0.917	0.750	0.786	0.750	0.700	0.690	0.717	0.700	0.600*
Gus	a	0.447	0.611*	0.438	0.375	0.417	0.380	0.350	0.370	0.375	0.390
	b	0.342	0.389*	0.354	0.411	0.333	0.380	0.400	0.413	0.425	0.540
	c	0.211	—	0.208	0.214	0.250	0.240	0.250	0.217	0.200	0.070
Hbdh-2	a	0.105	0.306	0.104	0.196	0.194	0.200	0.250	0.217	0.225	0.020
	b	0.895	0.694	0.896	0.804	0.806	0.800	0.750	0.783	0.775	0.980
Hk-2	b	0.447	0.694*	0.354	0.411	0.417	0.340	0.340	0.413	0.400	0.090
	c	0.553	0.306*	0.646	0.589	0.583	0.660	0.660	0.587	0.600	0.910
Idh-2	a	0.553	0.333*	0.542	0.500	0.528	0.520	0.480	0.500	0.450	0.440
	b	0.447	0.667*	0.458	0.500	0.472	0.480	0.520	0.500	0.550	0.560
Me	b	0.868	0.944	0.896	0.821	0.861	0.800	0.790	0.826	0.800	0.740
	c	0.132	0.056	0.104	0.179	0.139	0.200	0.210	0.174	0.200	0.260
Mpi	a	0.053	—	0.042	0.089	0.056	0.040	0.080	0.043	0.075	—
	b	0.447	0.750	0.417	0.375	0.472	0.440	0.370	0.413	0.450	0.530*
	c	0.395	0.222	0.396	0.411	0.333	0.360	0.370	0.413	0.375	0.420*
	d	0.053	0.028	0.083	0.125	0.083	0.080	0.120	0.087	0.050	0.050*
	e	0.053	—	0.063	—	0.056	0.080	0.060	0.043	0.050	—
Pgm	c	0.105	0.056	0.104	0.089	0.111	0.160	0.120	0.174	0.100	—
	d	0.658	0.528	0.688	0.625	0.639	0.520	0.540	0.522	0.600	0.720
	e	0.237	0.417	0.208	0.286	0.250	0.320	0.340	0.304	0.300	0.280
Xo	a	0.053	—	0.083	—	0.056	—	—	—	—	—
	b	0.947	1.000	0.917	1.000	0.944	1.000	1.000	1.000	1.000	1.000

*Significant deviation from H.-W. (sample codes as for Table 2).
The following electromorphs were fixed: *Acon-2* a, *Ak* c, *Est-1* a, *Got-1* a, *Got-2* b, *G6pd* b, *Hbdh-1* a, *Hbdh-3* a, *Hk-1* b, *Idh-1* b, *Mdh-1* a, *Mdh-2* c, *Pgam* b, *Xdh* b.

structuring among North Island populations apparently results from the differences of the C14 and C29 populations from the C8 and C37 populations.

Comparisons across all populations revealed significant heterogeneity at 10 out of 16 loci for type I&III populations and 12 out of 13 loci for type II populations (Table 8). The patterns of difference amongst type I&III populations essentially reflect those found amongst the North Island populations, in that most populations throughout the country differ only slightly, with the exception of the C14 and C29 populations. These two populations are not only quite different

Table 6 Electromorph frequencies in wild populations of *Ctenopseustis obliquana* type II

Locus	Electromorph	Sample codes							
		C3	C35	C23	C6	C21	C31	C34	C29
Acon-1	a	0.190	0.044	0.182	0.206	0.200	0.214	0.162	—
	b	0.810	0.956	0.818	0.794	0.800	0.786	0.838	1.000
Ada	a	0.357	0.511	0.394	0.412	0.400	0.429	0.365	—
	c	0.119	—	0.106	0.088	0.110	0.071	0.149	0.500
	d	0.524	0.489	0.500	0.500	0.490	0.500	0.486	0.500
Ao	a	0.214	0.511*	0.258	0.324	0.290	0.321	0.216	—
	b	0.786	0.489*	0.742	0.676	0.710	0.679	0.784	1.000
Est-2	a	0.929	1.000	0.909	0.912	0.870	0.911	0.919	0.848*
	b	0.071	—	0.091	0.088	0.130	0.089	0.081	0.152*
Gpi	a	0.476	0.256*	0.455	0.412	0.400	0.375	0.432	0.621*
	b	0.524	0.744*	0.545	0.588	0.600	0.625	0.568	0.379*
Gsr	b	0.048	—	0.045	0.088	0.120	0.089	0.068	0.182
	c	0.952	1.000	0.955	0.912	0.880	0.911	0.932	0.818
Gus	a	0.833	0.922	0.833	0.853	0.840	0.875	0.824	0.758*
	b	0.143	0.078	0.152	0.088	0.110	0.071	0.162	0.242*
	c	0.024	—	0.015	0.059	0.050	0.054	0.014	—
Hbdh-2	a	0.071	0.367*	0.061	0.118	0.160	0.089	0.054	—
	b	0.929	0.633*	0.939	0.882	0.840	0.911	0.946	1.000
Hk-2	b	0.214	—	0.182	0.235	0.210	0.268	0.176	0.242
	c	0.786	1.000	0.818	0.765	0.790	0.732	0.824	0.758
Mpi	b	0.310	0.089	0.303	0.235	0.230	0.214	0.297	0.364
	c	0.690	0.911	0.697	0.765	0.770	0.786	0.703	0.636
Pgam	a	0.024	—	0.045	—	—	—	0.054	—
	b	0.976	1.000	0.955	1.000	1.000	1.000	0.946	1.000
Pgm	c	0.119	—	0.167	0.118	0.110	0.089	0.135	—
	d	0.762	1.000	0.727	0.735	0.700	0.732	0.730	0.909
	e	0.119	—	0.106	0.147	0.190	0.179	0.135	0.091
Xo	a	0.119	0.289*	0.091	0.088	0.070	0.054	0.122	0.182*
	b	0.881	0.711*	0.909	0.912	0.930	0.946	0.878	0.818*

*Significant deviation from H.-W. (sample codes as for Table 2).
The following electromorphs were found to be fixed: *Acon-2* a, *Ak* c, *Est-1* a, *Fum* c, *Got-1*a, *Got-2* b, *G6pd* b, *α Gpd* c, *Hbdh-1* a, *Hbdh-3* a, *Hk-1* c, *Idh-1* b, *Idh-2* a, *Mdh-1* a, *Mdh-2* c, *Me* a, *Xdh* b.

from the other populations at the *Ada* locus, but they also differ from all other populations and considerably from each other for the *Hk-2* locus. The type II patterns are not dissimilar. Amongst the South Island and Stewart Island populations there is minor variation in electromorph frequencies at the *Ada* and *Ao* loci. However, the single North Island and Chatham Island populations are very different, both from the remaining populations and each other.

F-statistics

Analyses were carried out at five levels: site (population), region, species (pheromone type), genus (a cryptic complex) and total. The results clearly showed that there was low differentiation ($F_{ST} < 0.05$) at most loci and moderate differentiation ($0.05 < F_{ST} < 0.15$) at a few, for comparisons between populations within regions or species, or between regions within species. However, there is great or very great differentiation ($0.25 < F_{ST}$) at most loci for populations, regions or species within genera. Grouping populations into pheromone types removes by far the most heterozygosity amongst subpopulations and explains a high proportion of the total genetic variance within the Dugdale (1966) taxa.

Discussion

Biological status of pheromone types

The first objective of this genetic analysis was to determine if the genetic distinctness which identified different pheromone types in the laboratory-reared moths

Table 7 χ^2 heterogeneity analysis for populations of *Planotortrix excessana* type A and type B&C and *Ctenopseustis obliquana* type I&III from three geographical regions of New Zealand

	North Island			North South Island			South South Island		
Locus	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
P. excessana type A									
Acon-1	2.068	2	0.35556	0.953	8	0.99853	0.106	2	0.94828
Ada	3.253	1	0.07130	0.698	4	0.95158	0.014	1	0.90538
Ak	5.668	2	0.05879	0.376	8	0.99996	0.476	2	0.78819
Est-2	0.127	1	0.72184	2.567	4	0.63272	0.001	1	0.98128
Fum	0.000	0	1.00000	5.344	4	0.25382	0.044	1	0.83431
αGpd	3.060	1	0.08023	1.171	4	0.88290	0.557	1	0.45567
Gpi	3.516	2	0.17240	4.881	8	0.77020	0.212	2	0.89933
Gus	0.348	1	0.55522	0.927	4	0.92065	0.159	1	0.69039
Hk-2	1.318	1	0.25087	4.615	4	0.32916	0.384	1	0.53550
Me	3.429	1	0.06407	0.686	4	0.95305	0.036	1	0.84901
Mpi	0.336	2	0.84537	1.354	8	0.99487	0.168	2	0.91949
Pgam	7.811	1	0.00519**	3.183	4	0.52767	1.314	1	0.25162
Pgm	1.481	1	0.22357	1.051	4	0.90192	0.337	1	0.56136
P. excessana type B&C									
Acon-1	0.415	2	0.81277	5.699	8	0.68091	0.013	2	0.99337
Ada	0.251	1	0.61668	1.996	4	0.73656	0.033	1	0.85557
Ak	0.283	2	0.86820	1.499	8	0.99272	0.582	2	0.74759
αGpd	0.000	0	1.00000	1.103	4	0.89372	0.653	1	0.41908
Gpi	0.110	1	0.74010	0.385	4	0.98370	0.034	1	0.85437
Gus	0.015	1	0.90301	3.587	4	0.46482	0.770	1	0.38020
Hk-2	0.166	1	0.68328	1.156	4	0.88532	0.193	1	0.66026
Mdh-2	1.129	1	0.28809	4.808	4	0.30757	0.291	1	0.58967
Me	0.029	1	0.86374	0.108	4	0.99860	0.399	1	0.52757
Mpi	0.502	1	0.47872	2.768	4	0.59741	0.504	1	0.47780
Pgam	5.182	1	0.02282*	2.704	4	0.60850	0.819	1	0.36541
Pgm	0.178	1	0.67323	2.748	4	0.60076	0.007	1	0.93387
C. obliquana type I&III									
Acon-1	6.473	6	0.37236	0.082	2	0.95995	2.934	4	0.56896
Ada	22.099	6	0.00116**	3.389	2	0.18372	1.036	4	0.90431
Ao	26.017	3	0.00001***	0.000	1	1.00000	1.093	2	0.57906
Est-2	19.012	3	0.00027***	0.062	1	0.80403	0.284	2	0.86750
Fum	11.444	3	0.00955**	0.034	1	0.85309	0.756	2	0.68513
αGpd	26.139	6	0.00021***	0.082	2	0.95995	0.269	4	0.99172
Gpi	30.714	6	0.00003***	0.252	2	0.88169	1.192	4	0.87935
Gsr	13.463	3	0.00374**	0.914	1	0.33898	0.113	2	0.94522
Gus	19.822	6	0.00298**	0.034	2	0.98300	0.299	4	0.98986
Hbdh-2	24.226	3	0.00002***	0.115	1	0.73398	0.522	2	0.77039
Hk-2	51.620	3	0.00000***	0.011	1	0.91604	0.818	2	0.66428
Idh-2	5.068	3	0.16692	0.234	1	0.62876	0.220	2	0.89601
Me	10.892	3	0.01232*	0.070	1	0.79085	0.258	2	0.87903
Mpi	23.527	12	0.02357*	4.655	4	0.32452	3.018	8	0.93322
Pgm	15.282	6	0.01817*	0.069	2	0.96627	0.945	4	0.91808
Xo	10.512	3	0.01468*	0.000	0	1.00000	0.000	0	1.00000

*$P \leq 0.05$, **$P \leq 0.01$, ***$P < 0.001$.

Table 8 χ^2 heterogeneity analysis for populations of *Ctenopseustis obliquana* and *Planotortrix excessana* pheromone types from different geographical regions

C. obliquana type II from two regions

	South of the South Island			Stewart Island		
Locus	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Acon-1	0.151	1	0.69803	0.045	2	0.97775
Ada	0.249	2	0.88282	0.663	4	0.95577
Ao	0.001	1	0.98060	0.232	2	0.89033
Est-2	0.035	1	0.85186	0.815	2	0.66545
Gpi-1	0.207	1	0.64879	0.144	2	0.93045
Gsr	0.188	1	0.66456	0.487	2	0.78386
Gus	0.232	2	0.89060	0.673	4	0.95466
Hbdh-2	0.143	1	0.70568	1.641	2	0.44015
Hki-2	0.260	1	0.61020	0.678	2	0.71231
Mpi	0.019	1	0.89034	0.070	2	0.96576
Pgam	0.594	1	0.44080	0.000	0	1.00000
Pgm	0.145	2	0.93022	0.542	4	0.96929
Xo	0.002	1	0.96736	0.407	2	0.81584

P. excessana types from throughout New Zealand

	P. excessana type A			*P. excessana* type B&C			
Locus	χ^2	d.f.	*P*	Locus	χ^2	d.f.	*P*
Acon-1	51.778	18	0.00004***	*Acon-1*	11.962	18	0.84921
Ada	30.770	9	0.00032***	*Ada*	5.461	9	0.79244
Ak	37.090	18	0.00510**	*Ak*	9.084	18	0.95776
Est-2	12.563	9	0.18340	*αGpd*	8.080	9	0.52609
Fum	15.488	9	0.07838	*Gpi*	4.798	9	0.85153
αGpd	23.402	9	0.00535**	*Gus*	12.304	9	0.19673
Gpi	57.223	18	0.00001***	*Hk*	2.711	9	0.97467
Gus	39.462	9	0.00001***	*Mdh-2*	13.735	9	0.13205
Hk-2	27.870	9	0.00100***	*Me*	3.991	9	0.91202
Me	11.853	9	0.22174	*Mpi*	5.023	9	0.83231
Mpi	26.388	18	0.09122	*Pgam*	8.899	9	0.44668
Pgam	17.284	9	0.04444*	*Pgm*	16.600	9	0.05536
Pgm	17.746	9	0.03824*				

C. obliquana types from throughout New Zealand

	C. obliquana type I&III			*C. obliquana* type II			
Locus	χ^2	d.f.	*P*	Locus	χ^2	d.f.	*P*
Acon-1	19.704	18	0.34971	*Acon-1*	26.164	7	0.00047***
Ada	63.927	18	0.00000***	*Ada*	108.389	14	0.00000***
Ao	27.587	9	0.00112**	*Ao*	53.292	7	0.00000***
Est-2	40.736	9	0.00001***	*Est-2*	14.522	7	0.04264*
Fum	25.318	9	0.00264**	*Gpi*	22.531	7	0.00206**
αGpd	42.626	18	0.00090***	*Gsr*	21.327	7	0.00332**
Gpi	40.197	18	0.00196**	*Gus*	23.306	14	0.05547
Gsr	15.876	9	0.06953	*Hbdh-2*	63.668	7	0.00000***
Gus	29.592	18	0.04161*	*Hk-2*	26.298	7	0.00045***
Hbdh-2	29.981	9	0.00044***	*Mpi*	20.473	7	0.00463**
Hk-2	53.057	9	0.00000***	*Pgam*	17.101	7	0.01676*
Idh-2	6.226	9	0.71706	*Pgm*	47.794	14	0.00001***
Me	11.760	9	0.22716	*Xo*	28.322	7	0.00019***
Mpi	44.766	36	0.14994				
Pgm	26.001	18	0.09974				
Xo	30.232	9	0.00040***				

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

was consistent among wild populations. Based on the results, many populations were a mixture of congeneric pheromone types. The complete absence of any heterozygotes in these populations at the *Hbdh-3* locus for *P. excessana* pheromone types and at the *Hk-1* locus for *C. obliquana* pheromone types confirms that these types do indeed represent biological species in nature. This consequently supports the suggestion that they are defined by differences in their respective SMRSs, in which long-range pheromone signalling forms an important part.

Population structuring

The recognition concept recognizes two consequences of differences in SMRS for the genetic structuring of populations which are a mixture of two 'cryptic' species. The first is a high level of discontinuity over the population as a whole and the second is relative genetic continuity within each 'cryptic' species. All the major genetic structuring noted in the wild populations of leafrollers pivots on the division of the generic groupings into their composite pheromone types. Some additional structuring is evident at lower levels but is of a lesser scale and more restricted nature. The general patterns in the frequency distribution of electromorphs observed are also generally consistent with that recorded for the laboratory-reared populations.

There was a high degree of genetic stability in gene frequencies within each pheromone type across considerable distances which suggests that these moths have reproductively continuous distributions. This may be because of the ability of the moths to move reasonable distances during their life history or because of other processes, such as wind or repeated human introductions which could move individuals or egg batches across greater distances. There is no suggestion of discrete geographical subdivision within the pheromone types, or even differences across broad geographical regions.

Together, the two properties of heterogeneity across and relative homogeneity within pheromone types would have confirmed the biological species status of these groups even in the absence of a distinct genetic marker. However, in such a case the pheromone typing of individuals would have been necessary prior to genetic analysis. There was some location-specific differentiation of leafroller populations where no significant geographical discontinuity existed. These locations (P30, C29 and C37) were in highly modified areas with regular chemical control programmes and frequent habitat modification with cultivation practices. Such conditions could produce local population crashes, extinctions and habitat effects which could produce genetic differentiation of the type seen. All other collections were for populations in areas that were relatively undisturbed.

Biological status of some of the populations

The biological status of some of the populations was less clear. Dugdale (1990) expressed doubt over whether North Island *C. obliquana* type II can be considered part of the same taxonomic entity as the rest of the type II populations based on differences in the forewing to costal fold length ratio. The type II population from North Island certainly did prove genetically differentiated from the South and Stewart Island populations. However, this level of differentiation did not approach that observed among the pheromone types. In addition, a similar level of differentiation was recorded between the Chatham Island (C35) type II population and the remaining populations, although there is no suggestion that this represents a distinct biological or taxonomic type.

Dugdale (1990) also considered that this difference between North Island and South Island type II was insufficient to justify differentiating them taxonomically without additonal supporting evidence. Currently, such a separation is not supported by pheromone differences, mating trials or absolute genetic differences. Thus type II appears to be homogeneous from a biological perspective.

Dugdale (1990) has differentiated *P. excessana* type A into *P. octoides* on the Chatham Islands and *P. octo* elsewhere based on relatively minor morphological differences. The Chatham Island population shows some genetic differentiation from the mainland populations but at a much lower level than seen between the type A and B&C pheromone types. The differentiation observed would not be unexpected for a population of a species spatially, and as a consequence almost certainly reproductively separated from its parent population for some time. Thus a thorough comparison of the pheromone composition of the mainland and Chatham populations and mating trials between them would be required to resolve the question whether *P. octoides* is a separate biological entity.

The key issue in using the genetic data in this way is the focus on the biological implications of the data in terms of what they tell us about reproduction and mate recognition. Other authors have also used such data sets in this way (Green *et al.*, 1990; Comparini & Biasiolo, 1991; Eber *et al.*, 1991). Other studies which draw taxonomic conclusions from the degree of genetic differentiation between groups often attempt to use such data as a measure of divergence and thus, they argue, of species status. Steck (1991), for example, compared the F_{ST} values in *Anastrepha* and *Rhagoletis* to argue for the existence of cryptic species in the

former genus. This is essentially a taxonomic approach which identifies species on the basis of some arbitrary level of difference rather than as discrete cohesive biological entities.

This study illustrates the value of genetic data for the identification of biological species when correlated with other biological information, in this case pheromone differences of importance in mate recognition. Distinct genetic markers are most useful, but patterns of discontinuities and continuities can also provide invaluable information on the status of groups within sympatric populations.

Acknowledgements

This research was made possible by grants from the Auckland University Research Committee to the authors. Much assistance was also provided by the DSIR Entomology Division, Mt Albert. In particular, Dr Steven Foster helped a great deal by discussing the work in detail and by providing resources. Dr John Dugdale also helped by discussing a range of issues concerned with the taxonomy of leafroller moths. We thank the DSIR for providing a postgraduate scholarship to CSW. This contribution represents publication number 52 from the Evolutionary Genetics Laboratory, University of Auckland.

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