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Molecular evidence using enzyme and RAPD markers for sympatric evolution in British species of *Tetramesa* (Hymenoptera: Eurytomidae)

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Some species of the insect genus *Tetramesa* (Hymenoptera: Eurytomidae), which has a world-wide distribution, are morphologically very similar, both in the adult and larval stages. In the British Isles, there are 37 recorded species, all of which feed on grasses as larvae and are largely host specific. Some form galls on their hosts; others do not. We used a range of enzyme and random amplified polymorphic DNA (RAPD) markers to investigate a complex of five cryptic species occurring sympatrically in the UK, collected from seven sites in mainland England and Wales: *T. calamagrostidis* (von Schlechtendal), *T. longicornis* (Walker) and *T. petiolata* (Walker) infesting different grass hosts, and *T. hyalipennis* (Walker) *s.l.* comprising two-host adapted forms (labelled 1 and 2) reared from the grasses *Elymus repens* and *E. farctus*, respectively. Nine soluble enzyme systems (some known to be polymorphic in other insects) and 37 RAPD primers allowed taxonomic separation of the species. However, whilst RAPD markers were able to discriminate between the two host-adapted forms of *T. hyalipennis*, enzyme markers (producing phenotypic profiles in the absence of genetic crosses) could not. Upon calculating genetic distances for the RAPD data from which a cladogram of Euclidean distances (relatedness) was produced along with multivariate analysis of the data, *T. longicornis* was shown to be the most 'basal' species, and most related to *T. hyalipennis s.l.; T. calamagrostidis* and *T. petiolata* were found to be more distantly related to these species but most closely related to each other. The two forms of *T. hyalipennis s.l.* appear to be the most closely related of any of the species investigated, probably diverging the most recently. From this data, and since the populations examined were all sympatric without obvious physical barriers to reproduction, it can be concluded that some degree of sympatric evolution has occurred, most obviously in the case of the host-adapted forms of *T. hyalipennis*. If so, this complex of species could be another rare example of sympatric speciation in insects. Further research using more sophisticated molecular markers such as microsatellites, amplified fragment length polymorphic markers (AFLPs) and DNA sequencing (e.g. of mtDNA and ribosomal DNA regions), in conjunction with behavioural studies, are required to further elucidate this interesting species group.

ADDITIONAL KEYWORDS: cladogram – enzymes – jointworms – multivariate analysis – RAPDs – sympatric speciation.

INTRODUCTION

Insects of the genus *Tetramesa* Walker (= *Isosoma* Walker; = *Harmolita* Motsch.) (Hymenoptera: Eurytomidae) are small (~2–3 mm in length), winged, generally black in colour, and feed on nectar as adult wasps. In the larval stage, they are phytophagous on cereals and grasses (Poaceae), the eggs being laid on the stems of their hosts (Claridge & Dawah, 1994). They are primarily a north temperate genus, comprising around 205 species world-wide (Holarctic, Afro-tropical; Burks, 1971, 1979). There are 63 species recorded from

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America, north of Mexico (Peck, 1963), and they are similarly abundant in the Palaearctic region with 61 species recorded from the former Soviet Union (Zerova, 1976, 1978) and 37 species from the British Isles (Fitton *et al*., 1978; see also Claridge, 1961; Claridge & Dawah, 1994). Some species form distinct galls on the stem of their hosts [e.g. *T. calamagrostidis* (von Schlechtendal)] or flowering parts [e.g. *T. hyalipennis* (Walker)], whilst others live singly in or above the nodes of flowering stems [e.g. *T. longicornis* (Walker)], with no real evidence of a galling habit (Claridge, 1961). Moreover, in some species the galls include a single chamber occupied by one larva [e.g. *T. brevicornis* (Walker)], whereas in others they include a sequence of several chambers each occupied by a single larva (e.g. *T. calamagrostidis)*. *Tetramesa* are extremely host-specific and previous studies have shown that they normally attack plant hosts of only one genus (Phillips, 1936; Claridge, 1961), although Dawah (1987) found *T. eximia* (Giraud) to attack hosts of two closely related genera, *Calamagrostis epigejos* (L.) Roth. and *Ammophila arenaria* (L.) Link.

Because some gall-making *Tetramesa* spp., such as *T. tritici* (Fitch), reduce the productivity of the flowering head and seed weight (Claridge, 1961; Spears, 1978), they are sporadic serious pests of cereals and grasses in various countries around the world, especially North America, where they are known as jointworms or strawworms (Phillips, 1927; Spears, 1978; Spears and Barr, 1985). They are not however, considered especially important in the UK (Claridge, 1961; see also http://www.nhm.ac.uk/entomology/chalcidoids/eurytomidae.html). Even so, and because of their general economic importance globally, they have been intensively studied in terms of their biology, ecology and taxonomy. Taxonomic difficulties as mentioned by Henneicke, Dawah & Jervis (1992), based on the extremely uniform morphology of adult wasps, has stimulated taxonomists to look for other discriminating criteria. These include host plant preference, mate choice and mode of larval life as well electrophoretic banding profiles to assess the status of very closely related species (Dawah, 1987; Claridge & Dawah, 1994).

An example of the problems encountered within the genus *Tetramesa* is well illustrated by *T. hyalipennis*. Hedicke (1920) morphologically separated *T. hyalipennis* emerging from *Elymus* (= *Agropyron*) *repens* (L.) Gould, Madrono and that emerging from *E. farctus* (Viv.) Runemark ex Melderis (which he misidentified as *A. arenaria*), on the grounds of an apparent different host-plant preference, and gave them subspecific status. Bailey (1967) supported Hedicke's subspecific separation of the two populations based on experiments on their host-plant specificity, differences in shape, size, egg production and sex ratios. Even so, Dawah (1987) found no differences between *T. hyalipennis* reared from *E. repens* and those reared from *E. farctus* using enzyme electrophoresis, host preferences and mate choice experiments. He suggested that *T. hyalipennis* reared from *E. repens* and *E. farctus* should be considered a single oligophagous species. Clearly, there are contradictory views on the taxonomic status of *Tetramesa* reared from *E. repens* and *E. farctus*.

The aim of this study was to explore further the taxonomic affinities of closely related, morphologically similar *Tetramesa* species living sympatrically in Britain using electrophoretic approaches: protein (allozyme markers) and DNA [random amplified polymorphic DNA markers amplified using the polymerase chain reaction (RAPD-PCR markers)]. The insects studied included the species complex *T. calamagrostidis, T. longicornis, T. petiolata* (Walker) and *T. hyalipennis s.l.* found on different grass hosts [e.g. *T. calamagrostidis* reared from *C. epigejos, T. longicornis* from *Phalaris arundinacea* L., *T. petiolata* from *Deschampsia caespitosa* (L.) Beauv. and *T. hyalipennis* from two closely related grass species, *E. repens* and *E. farctus*]. By so doing, it was hoped to demonstrate that even species that may in effect be cryptic sibling species, differentiable only as a result of larval host-feeding preferences, could be taxonomically discriminated using genetic markers. We also considered that these forms could have arisen originally by a process of sympatric speciation.

MATERIAL AND METHODS

GRASSES

Grasses were collected in the autumns and late winters of 1997–2000 from localities in South Wales and England (Fig. 1). Sites were identified according to their county (Local Government Act 1972) and National Grid Reference (NGR). (See Al-Barrack, 2001, for a description of each site, including floral composition): 1. Magor (Monmouth County Borough Council; NGR: ST 425 865); 2. Fairwater (City and County of Cardiff; NGR: ST 133 788); 3. Cosmeston lakes country park (Vale of Glamorgan; NGR: ST 174 613); 4. Merthyr Mawr sand dunes (Bridgend County Borough Council; NGR: SS 860 770); 5. Kenfig Dunes (Bridgend County Borough Council; NGR: ST 794 816); 6. Caerphilly (Caerphilly County Borough Council; NGR: ST 156 852); 7. Hell Coppice (Oxfordshire; NGR: SP 608 104); 8. Oddington (Oxfordshire; NGR: SP 555 145); 9. Wren's Nest Hill (Dudley, West Midlands; SO 938 919).

INSECTS

For both enzyme and RAPD-PCR studies, about 30 female individuals of each species were tested electro-

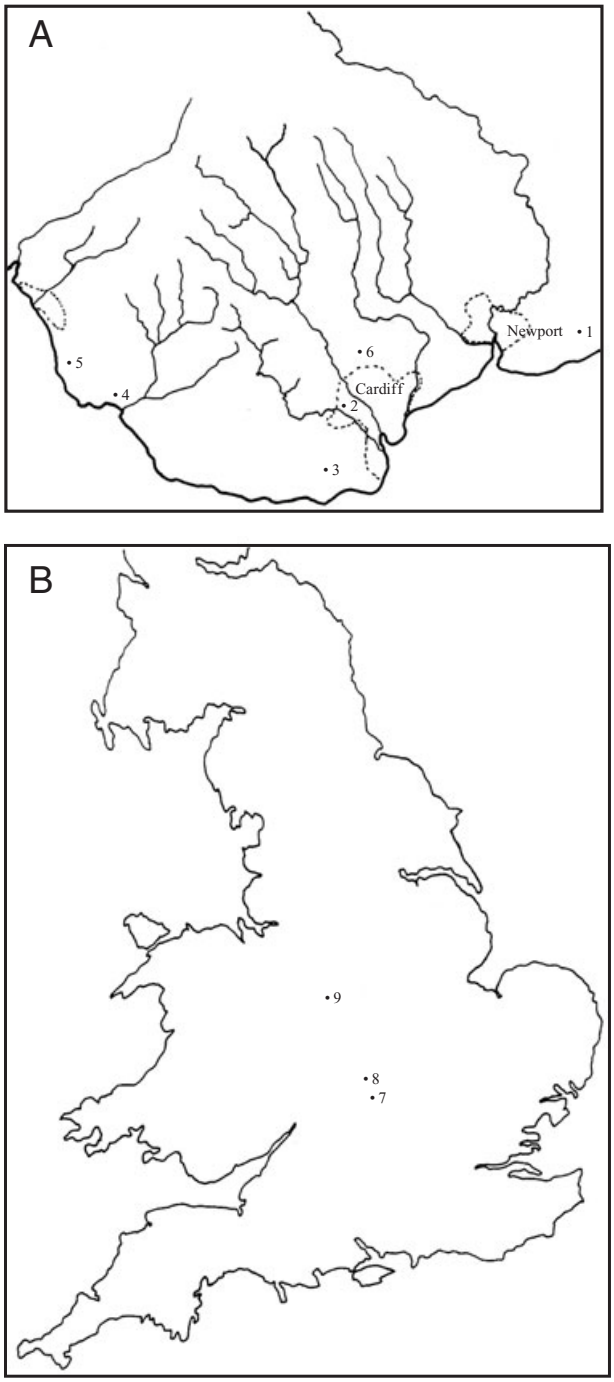

Figure 1. A, collecting sites for *Tetramesa* in South Wales. 1 = Magor; 2 = Fairwater; 3 = Cosmeston lakes country park; 4 = Merthyr Mawr dunes; 5 = Kenfig Dunes; 6 = Caerphilly. B, Collecting sites for *Tetramesa* in England. 7 = Hell Coppice (Oxfordshire); 8 = Oddington (Oxfordshire); 9 = Wren's Nest Hill, Dudley, West Midlands.

phoretically. *Tetramesa* sp. were dissected as larvae from their respective grass hosts and reared as described in detail by Graham & Claridge (1965). All adults were kept outdoors for 10 days to ensure the complete hardening of the cuticle and attainment of adult colour and then stored at –80 °C prior to electrophoretic testing. Due to time constraints, no genetic crosses were performed in this study to verify the enzyme and RAPD bands observed.

CHEMICALS/BIOCHEMICALS

Chemicals and other reagents used were of high purity grades and purchased from BDH and Sigma (Poole, Dorset) with the exception of *Taq* polymerase and preprepared PCR reaction buffer and $MgCl_2$ solutions, bought from Bioline (Humber Road, London). All solutions were made up in de-ionized water, the pH being adjusted as necessary using HCl or NaOH.

ENZYME ELECTROPHORESIS

i) Sample preparation

Specimens were homogenized individually in 50 μL ice-cold homogenizing buffer (Loxdale, Castañera & Brookes, 1983): 15% (w/v) sucrose, 50 mM Tris-HCl, pH 7.1 in 0.5% (v/v), Triton X-100). For electrophoresis, we used the cellulose acetate electrophoresis system, including horizontal tank, applicator, applicator base plate and cellulose acetate plates, supplied by Helena Laboratories, Beaumont, Texas, USA (see Wynne, Loxdale & Brookes, 1992 for details, including the many advantages of using this particular system for enzyme electrophoresis). All practical work was performed at Rothamsted Research, Harpenden, UK. Multiple aliquots of cryogenically preserved homogenates suitable for enzyme electrophoresis were prepared and stored long-term in liquid N_2 as described by Wynne & Brookes (1992). For enzymes with high activity, such as phosphoglucose isomerase (PGI) (EC 5.3.1.9), only one application (0.25 μL) was required; with other enzymes, several applications were needed to achieve reasonable staining. Electrophoresis was performed at a constant voltage (200 V) for 30 min. Due to the invariance of the enzymes tested from *T. hyalipennis* reared from *E. farctus* (see Results), specimens of this species were always run on lanes 9 and 10 as mobility reference standards.

ii) Running and staining

The recipes used for the 19 enzymes tested (see below and Al-Barrak, 2001 for other details) were taken mainly from Richardson, Baverstock & Adams (1986), Wynne *et al.* (1992), whilst in the case of esterases, we used 20 mg Fast Blue RR salt, 2 mL 0.5 M Tris/HCl, pH 7.1, 8 mL H_20; filter and to 2 mL of this, add

200 μL 1% (w/v) α- or β-naphthyl acetate dissolved in 50% (v/v) methanol-water. Plates were run using two electrophoretic running buffers: (1) 'TC buffer', 50 mM Tris-citrate, pH 7.8, or (2) 'TG buffer', 25 mM Tris-glycine, pH 8.5. Usually the TC buffer was used, but occasionally both buffers were employed in different runs, as specified in the text and figures.

The enzymes tested were soluble proteins known to show good staining and resolution and often polymorphisms (electromorph mobility) in studies of other small insects: aconitate hydratase (ACON, EC 4.2.1.3); acid phosphatase (ACP, EC 3.1.3.2); aldolase (ALD, EC 4.1.2.13); aldehyde oxidase (AO, EC 1.2.3.1); α-esterase (α-EST, EC 3.1.1); β-esterase (β-EST, EC 3.1.1); glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49); glycerol-3-phosphate dehydrogenase (α-GPD, EC 1.1.1.8); β-hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30); hexokinase (HK, EC 2.7.1.1); isocitrate dehydrogenase (IDH, EC 1.1.1.42); lactic dehydrogenase (LDH, EC1.1.1.27); malate dehydrogenase (MDH, EC 1.1.1.37); malic enzyme (ME, EC 1.1.1.4); mannose-phosphate isomerase (MPI, EC 5.3.1.8); PGI; 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44); phosphoglucomutase (PGM, EC 2.7.5.1); and L-sorbitol dehydrogenase (SORDH, EC 1.1.1.14).

Staining was performed as described in Wynne *et al.* (1992): 2 mL staining reaction mixture and 2 mL hot agar solution at ~60 °C were mixed and then poured immediately onto the gel in a single action. The cellulose acetate plate was quickly tilted to produce a uniform thin covering of the mixture. This was then left to set for a few minutes, after which the plates were left in the dark [stains containing phenazine methosulphate (PMS) and methyl-thiozyl blue (MTT) only], until the bands appeared (usually within a few minutes, but with esterase, half an hour). Following the appearance of bands, plates were fixed using 7% (v/v) glacial acetic acid. The stain buffer in all recipes was 0.1 M Tris-HCl, pH 8.0. The concentrations of stock solutions were as follows: NADP/NAD = 20 mg/mL; 1.0 M $MgCl_2$ = 95 mg/mL; 0.2 M $MgCl_2$ = 19 mg/mL; PMS = 2 mg/mL; MTT = 6 mg/mL.

iii) Interpretation of enzyme electrophoretic data

The criteria we used for assessing variation between *Tetramesa* species were: (1) number, (2) position and (3) density of bands on the gel. These provided an approximate estimate of the relative amount of protein in each sample along with the comparative enzyme mobilities for each species on a number of gels. There are two methods of analysing phenotypic and genotypic electrophoretic data. The phenotypic method was used, i.e. we compared gross phenotype profiles rather than scoring allele and genotype frequencies directly following gel running. This method involved measuring the relative mobility (Rm) of the bands in millimetres from the sample origin. Considering the objective of this study, that is, the investigation of taxonomic differences between *Tetramesa* species, the phenotypic approach was found to be sufficient to answer the questions raised from the objectives (see also Statistical analysis).

RAPD-PCR

i) DNA extraction and purification

Adult wasps only were used for DNA extraction. Samples were kept on ice in 0.5 mL-Eppendorf tubes containing 297 μL TNES buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM EDTA; 1 mM sodium orthovanadate; 1% (v/v) 'Nonidet P40'). Proteinase K (3.0 μL) was than added to each sample followed by homogenization using a Miscraft drill for a few seconds. The tubes were incubated in a thermocycler (Hybaid) for 3–18 h or overnight at 37 °C. 85 μL 5.0 M NaCl was added and shaken using a vortex machine (Auto vortex, SAS Stuart Scientific). Samples were then centrifuged for 5 min at 14 000 r.p.m. The supernatant was decanted into a 1.5-mL clean Eppendorf tube using a micropipette, care being taken to prevent the tip of the micropipette from touching the pellet. The pellet was suspended in absolute alcohol previously cooled to –20 °C for 1 h. The mixture was then shaken for 2 min and the tubes left to warm to room temperature. They were centrifuged for 20 min at 14 000 r.p.m. to detect the presence of DNA in the tube. The supernatant and DNA were carefully separated, and the DNA was resuspended in 70% ethanol at –20 °C and centrifuged for a further 10 min at 14 000 r.p.m. The ethanol was then removed with a micropipette, and a tissue wick was used to carefully absorb the remainder. Tubes were left to dry completely as the ethanol evaporated. Lastly, the DNA was resuspended with 20–50 μL water followed by a short centrifugation, whereafter the tubes were put in the fridge at ~4 °C overnight. They were shaken vigorously before use. To check the DNA concentration, three 0.5-mL Eppendorf tubes of different serial DNA concentrations ($\times 10, \times 100$ and $\times 1000$ dilution) were prepared and the optimal DNA concentration was selected in terms of product intensity at a wavelength of 260 nm (see http://www.mcrc.com/quantifyingDNA.htm).

ii) Preparation of the agarose gel and gel loading

We added 2 g agarose to 200 mL 0.5 × TBE (45 mM Tris-borate: 1 mM EDTA buffer, pH 8.0) in an Erlenmayer flask, and heated it in a microwave oven for 3 min until it was completely dissolved and clear. It

was then left to cool to 60 °C. 2 μL ethidium bromide was then added, the mixture shaken and the gel slowly poured into the electrophoresis tank. The open side of the tank was closed using sticky tape, care being taken to remove the bubbles in the gel mixture. Toothed sample combs were inserted at a distance of 1 cm from the edge and in the middle of the gel, which was then left for 1–2 h to set. The combs were then removed and the gel tray with gel placed in the electrophoresis tank and submerged in 0.5 × TBE buffer (4–5 mm). 2 μL ethidium bromide was included in the buffer at the anodal end of the tank.

For sample preparation, samples (10 μL PCR product) were mixed with 2 μL gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) using a micropipette (one tip per sample) in slight depressions made in Parafilm placed over an ELISA plate. Samples were then loaded in each gel well using the same micropipette tip previously used for mixing.

iii) Primers and PCR reaction mix

Arbitrary RAPD primers (10-mers) were purchased from Operon Technologies, Inc. (Alameda, CA 94501, USA). On arrival, they were resuspended in water according to the product instructions (50 ng μL^{-1}). The PCR reaction buffer for one reaction contained: 19.375 μL H_2O; 2.5 μL 10 × NH_4 reaction buffer; 0.75 μL $MgCl_2$ (50 mM); 1.00 μL primer (50 ng μL^{-1}); 0.25 μL dNTP mix (20 mM); 0.125 μL *Taq* polymerase (500 U pack); 1.00 μL template DNA = 25 μL total per tube. For each sample, the DNA concentration was calculated. On addition of the primer and *Taq* to the tube, it was thoroughly shaken. Samples were always kept on ice.

A drop of mineral oil was layered over each sample to prevent evaporation. All samples were then transferred to the thermal cycler and the appropriate programme selected. PCR cycle conditions used were as follows: 32 cycles at 92, 35 and 72 °C each of 1 min duration, except for the first denaturing step of the first cycle which was 2 min and the extension step of the last cycle which was 5 min. At the end of the program, samples were run on a gel or transferred to a refrigerator to be tested later. A 123-bp DNA ladder was used in all electrophoretic runs as a molecular size marker (Gibcorbal, USA, lot no. KK9706). The ladder was diluted as follows: 25 μL ladder, 175 μL H_2O and 50 μL gel loading buffer. Gels were run at a constant voltage of 80–100 V, for 3–3.5 h, whereafter the gel tray was removed and the gel visualized and then photographed on a UV trans-illuminator. Fifty-five primers were screened in total (Appendix; see below) and the banding patterns evaluated according to Puterka *et al*. (1993). The dominant RAPD bands produced (Loxdale & Lushai, 1998) were arbitrarily ascribed to particular loci and the presence or absence of amplification products recorded in a matrix of 1 s or 0 s.

Statistical analysis

Enzyme banding patterns ('phenograms') were compared visually. ANOVA was performed on the Rms for each 'useful' enzyme band identified per species, although a cladogram of genetic distances was not produced because of the difficulty in ascribing enzyme bands of complex phenotypes to particular genetic loci.

For RAPD analysis, primer sequences and number of bands scored and their respective degree of polymorphism are listed in the Appendix. Only primers that gave scorable bands (diagnostic or shared) were analysed. Evaluation of the primers was based not only on the degree of separation or amplification of bands, but also on differences in molecular sizes (bp) of the amplification products from the *Tetramesa* species studied. The greater the observed differences in band size, the more polymorphism was seen to be diagnostic between species.

The RAPD fingerprints generated for the *Tetramesa* species were analysed by RAPDPLOT 2.4 (Black, 1996) to generate Euclidean genetic distances. This is probably the most commonly chosen type of distance. It is simply the geometric distance in multidimensional space and is computed as: $\text{distance}(x,y) = (\Sigma_I (x_i - y_i)^2)^{1/2}$. Thereafter cluster and multivariate analyses – multidimensional scaling (MDS) and factor analysis – were performed to verify that the interpretation of the data did not depend on the type of analysis used. The analysis was performed using the Statistica 4.0 program from Statsoft Inc., USA and run in an IBM 'Windows 95' environment. MDS analysis, which can be considered as an alternative to factor (discriminant) analysis, uses a function minimization algorithm that evaluates different configurations with the goal of maximizing the goodness-of-fit. A coefficient called 'Stress' is used to determine the reliability of the MDS plot (Kruskal, 1964a, b). The most common measure that is used to evaluate how well (or poorly) a particular configuration reproduces the observed distance matrix is the Stress measure. Thus, the smaller the Stress value, the better the fit of the reproduced distance matrix to the observed distance matrix. Finally, we used factor analysis as a classification method, performed using the RAPD data (presence/absence) with the following conditions: Rotation: unrotated; Extraction: principal components.

RESULTS

Of the 19 enzyme systems tested, only nine showed distinct, well-resolved and repeatable electrophoretic banding patterns: α-EST, β-EST, G-6-PDH, HK, IDH, ME, MDH, PGI and PGM. A summary of the electrophoretic results is shown in Table 1, whilst the electrophoretic banding patterns for two enzymes, IDH and PGI are shown in Figure 2. The ANOVA of the relative mobilities for each of the enzymes tested (not shown) revealed that all the chosen enzymes studied displayed significant differences between species, with the exception of MDH, which was monomorphic (see below).

ENZYMES

Below are descriptions of phenotypic profiles for species enzyme bands tested [band relative mobility values (mm) are given in parentheses].

EST

No heterozygotes were observed in any of the samples analysed. Two distinctive bands with different mobilities were always revealed. Analysis of α- and β-

Table 1. Summary of enzyme electrophoretic results for *Tetramesa* sp. (mean distances travelled in mm). *TC, Tris-citrate pH 7.8; **TG, Tris-glycine pH 8.5; *N*, sample size; SD, standard deviation of mean; SE, standard error of mean

		Enzyme					
Species		α-EST	β-EST	G-6-PD	HK	IDH-TC*	IDH-TG**
T1	Mean	30.5	36.5	8.7	15.8	18.7	9.8
T. calamagrostidis	*N*	30	30	30	30	30	30
	SD	4.57	5.59	2.17	3.64	4.43	6.59
	SE	0.84	1.00	0.40	0.66	0.81	1.21
	Min.	26.0	31.0	6.0	8.0	13.0	4.0
	Max.	35.0	42.0	12.0	19.0	25.0	28.0
T2	Mean	25.5	32.5	8.70	22.5	24.7	17.5
T. longicornis	*N*	30	30	30	30	30	30
	SD	5.59	4.58	2.17	3.62	4.91	7.07
	SE	1.02	0.84	0.40	0.66	0.89	1.29
	Min.	20.0	28.0	6.0	15.0	18.0	10.0
	Max.	31.0	37.0	12.0	26.0	32.0	34.0
T3	Mean	31.0	40.0	8.70	23.8	17.8	13.5
T. petiolata	*N*	30	30	30	30	30	30
	SD	6.10	5.08	2.17	1.23	5.10	4.89
	SE	1.11	0.93	0.40	0.22	0.93	0.89
	Min.	25.0	35.0	6.0	22.0	8.0	9.0
	Max.	37.0	45.0	12.0	26.0	25.0	25.0
T4	Mean	19.5	36.0	3.3	7.0	18.7	13.9
T. hyalipennis	*N*	30	30	30	30	30	30
(ex: *E. repens*)	SD	3.56	2.03	1.40	0.83	5.11	3.22
	SE	0.65	0.37	0.25	0.15	0.94	0.59
	Min.	16.0	34.0	2.0	6.0	9.0	10.0
	Max.	23.0	38.0	6.0	8.0	26.5	22.0
T5	Mean	25.0	38.0	3.30	7.0	18.7	13.9
T. hyalipennis	*N*	30	30	30	30	30	30
(ex: *E. farctus*)	SD	9.15	4.07	1.40	0.83	5.11	3.22
	SE	1.67	0.74	0.25	0.15	0.93	0.59
	Min.	16.0	34.0	2.0	6.0	9.0	10.0
	Max.	34.0	42.0	6.0	8.0	26.5	22.0
Total	Mean	26.3	36.6	6.5	15.2	19.7	13.7
	N	150	150	150	150	150	150
	SD	7.35	5.04	3.22	7.65	5.50	5.73
	SE	0.60	0.41	0.26	0.62	0.45	0.47
	Min.	16.0	28.0	2.0	6.0	8.0	4.0
	Max.	37.0	45.0	12.0	26.0	32.0	34.0

Table 1. *Continued*

Species		Enzyme					
		ME-TC*	ME-TG**	MDH	PGI-TC*	PGI-TG**	PGM
T1	Mean	30.6	31.7	12.0	12.1	1.8	11.2
T. calamagrostidis	*N*	30	30	30	30	30	30
	SD	3.70	1.73	8.14	4.21	3.70	6.40
	SE	0.67	0.31	1.48	0.77	0.67	1.17
	Min.	25.0	30.0	4.0	5.0	0.0	6.0
	Max.	35.0	34.0	20.0	20.0	15.0	20.0
T2	Mean	30.6	31.7	12.0	20.3	8.5	11.2
T. longicornis	*N*	30	30	30	30	30	30
	SD	3.67	1.73	9.15	8.84	5.95	6.39
	SE	0.67	0.31	1.67	1.61	1.08	1.17
	Min.	25.0	30.0	3.0	11.0	0.0	6.0
	Max.	35.0	34.0	21.0	40.0	25.0	20.0
T3	Mean	30.6	31.7	12.0	15.5	6.7	12.8
T. petiolata	*N*	30	30	30	30	30	30
	SD	3.67	1.73	8.14	3.69	5.85	6.39
	SE	0.67	0.31	1.48	0.67	1.06	1.17
	Min.	25.0	30.0	4.0	9.0	2.0	6.0
	Max.	35.0	34.0	20.0	21.0	25.0	20.0
T4	Mean	29.0	29.7	12.0	8.2	7.3	6.0
T. hyalipennis	*N*	30	30	30	30	30	30
(ex: *E. repens*)	SD	4.11	1.72	8.13	4.09	1.82	6.51
	SE	0.75	0.32	1.48	0.74	0.33	1.19
	Min.	23.0	28.0	4.0	2.0	5.0	1.0
	Max.	34.0	32.0	20.0	14.0	10.0	15.0
T5	Mean	29.0	29.7	12.0	8.2	7.3	6.0
T. hyalipennis	*N*	30	30	30	30	30	30
(ex: *E. farctus*)	SD	4.12	1.73	8.14	4.09	1.82	6.51
	SE	0.75	0.31	1.48	0.75	0.33	1.18
	Min.	23.0	28.0	4.0	2.0	5.0	1.0
	Max.	34.0	32.0	20.0	14.0	10.0	15.0
Total	Mean	29.7	30.9	12.0	12.9	6.3	9.4
	N	150	150	150	150	150	150
	SD	3.88	1.97	8.24	7.03	4.79	6.98
	SE	0.32	0.16	0.67	0.57	0.39	0.57
	Min.	23.0	28.0	3.0	2.0	0.0	1.0
	Max.	35.0	34.0	21.0	40.0	25.0	20.0

esterases from each species studied allowed differentiation of the populations studied.

α-EST

The highest Rm was found in *T. petiolata* (31) followed by *T. calamagrostidis* (30). The lowest value was found in *T. hyalipennis* (ex: *E. repens*) (19.5) (Table 1). Note that *T. hyalipennis* (ex: *E. farctus*) always showed a higher level of enzyme activity than did *T. hyalipennis* (ex: *E. repens*).

β-EST

The highest Rm was found in *T. petiolata* (40) followed by *T. hyalipennis* (ex: *E. farctus*) (38), *T. calamagrostidis* and *T. hyalipennis* (ex*: E. repens*) (36), and the lowest value in *T. longicornis* (32.5) (Table 1). As with α-EST, *T. hyalipennis* (ex: *E. farctus*) always showed higher levels of enzyme activity than did *T. hyalipennis* (ex: *E. repens*) (not shown).

G-6-PDH

G-6-PDH occurred as a single invariant (monomorphic) band in each of the species studied, as found earlier by Dawah (1987). *T. calamagrostidis*, *T. longicornis* and *T. petiolata* had the same Rm of 8.7, while both *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) had the same Rm of ~3.0.

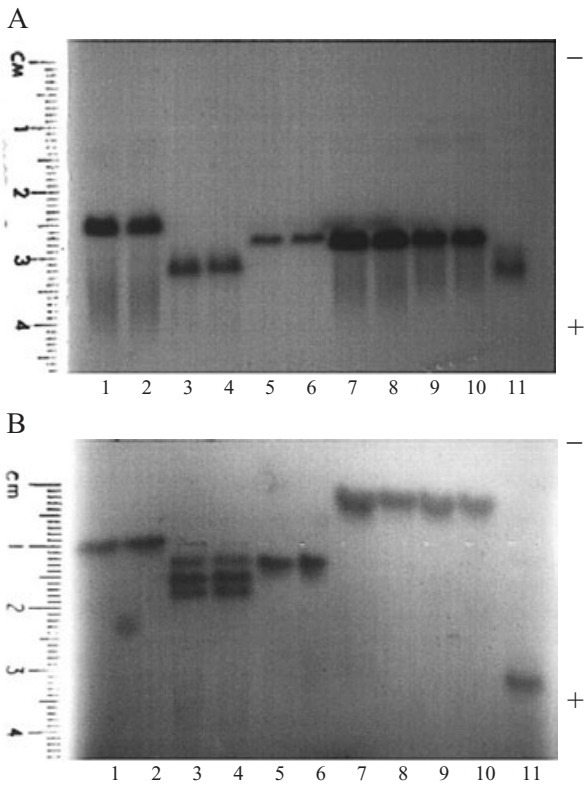

Figure 2. Electrophoretic banding patterns for (A) isocitrate dehydrogenase, IDH, and (B) phosphoglucose isomerase, PGI. Lanes 1,2, *T. calamagrostidis*; 3,4, *T. longicornis*; 5,6, *T. petiolata*; 7,8, *T. hyalipennis* from *E. repens*; 9,10, *T. hyalipennis* from *E. farctus*; 11, control. The arrowhead shows the starting point. – indicates the cathode and + the anode.

HK

The hexokinase pattern was polymorphic in the *Tetramesa* species studied. In *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*), a band with the same Rm value of 7.0 was resolved, which differed from the bands of *T. calamagrostidis*, *T. longicornis and T. petiolata* (15.8, 22.5 and 23.8, respectively). Doublets were seen in lanes 3, 4, 7 and 8, which could have been monomeric heterozygotes (Richardson *et al.*, 1986).

IDH (Fig. 2A)

IDH produced a single monomorphic band in each of the species studied, as found earlier by Dawah (1987). For this enzyme, two buffers were used – TC and TG. The highest Rm value was found in *T. longicornis* (24.7 TC) and (17.5 TG) followed by *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) (18.7 TC and 13.9 TG, respectively) and *T. calamagrostidis* (18.7 TC, 9.8 TG), with *T. petiolata* having the lowest value (17.8 TC, 13.5 TG).

ME (NADP⁺ specific) (Richardson *et al.,* 1986)

ME showed as a single monomorphic band in all the species tested. *T. calamagrostidis, T. longicornis* and *T. petiolata* each had the same Rm with buffers TC and TG, of 30.6 and 31.7, respectively. *T. hyalipennis* ex: *E. repens* and ex: *E. farctus* had the same value with each buffer of 29.0 and 29.7, respectively.

MDH (NAD⁺ specific) (Richardson *et al.,* 1986).

One invariant band was found between the species tested.

PGI (Fig. 2B)

For this enzyme, both TC and TG buffers were used. *T. calamagrostidis* and *T. petiolata* had Rms of 12.1 (TC) and 15.5 (TG), respectively. *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) had one band, but its mobility was in the opposite direction of migration, i.e. cathodally. In contrast, *T. longicornis* had three bands, which differed radically from the banding profiles of other species and were possibly dimeric heterozygotes (Richardson *et al.*, 1986).

PGM

PGM was monomorphic in all the species studied. *T. petiolata* showed the highest Rm (12.8) followed by *T. calamagrostidis* and *T. longicornis* (11.2), with *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) having the same value of 6.0.

In summary, nine enzymes stained well and provided diagnostic bands and differences in their relative mobilities (Table 1), allowing separation of *T. calamagrostidis*, *T. longicornis*, *T. petiolata* and *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) combined. No electrophoretic mobility differences were found between the two forms of *T. hyalipennis*, although staining intensity differences in α- and β-EST were noted. Adaptation to environmental differences could be responsible for these differences. Thus the taxonomic status of *T. hyalipennis* remained unclear using enzyme markers alone, as previously noted by Dawah (1987).

RAPDs

The amplified products of *Tetramesa* species *T. calamagrostidis, T. longicornis, T. petiolata* and *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*), obtained using a range of arbitrary 10-mer primers, were screened electophoretically. The appendix shows the different RAPD-PCR profiles obtained with each primer.

Of the 55 primers screened, 18 failed to give any products for reliable interpretation, whilst 37 primers gave clear and consistent differences between

T. calamagrostidis, T. longicornis, T. petiolata and *T. hyalipennis* in terms of number of polymorphic bands. However, of these 37 only eight (OPA5, UB2, OPG19, OPF3, OPF4, OPF7, OPM1, OPM15) gave variable and inconsistent products for the host-associated population of *T. hyalipennis* (ex: *E. repens* and ex *E. farctus*). Therefore, the differences between the two forms of *T. hyalipennis* were not diagnostic using these particular primers. Even so, the statistical analysis of host-associated populations of *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) showed slight separation (see below) and primers OPA5 (not shown) and OPG19 (Fig. 3A) were the most suitable generally for use in taxonomic discrimination of *Tetramesa* species. Examination of intraspecific variation revealed that out of eight of the primers tested (OPA2, OPA5, UB1, OPG19, OPF4, OPM7, OPM15, OPM19), only two showed sufficient variation for useful diagnostic bands: OPG19 and OPF4. The remainder gave monomorphic RAPD banding profiles per species. OPG19 showed variation within *T. longicornis* (8 bands/14 individuals) and *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*) 3 bands/18 individuals). With OPF4, intraspecific variation was found in individuals of *T. calamagrostidis* (1 band/20 individuals), *T. petiolata* (2 bands/20 individuals) and *T. hyalipennis* (ex: *E. repens* and ex: *E. farctus*; both with 3 bands/20 individuals), yet the primer was broadly diagnostic (Fig. 3B). With primer OPM15, all the species could be distinguished, including the two host-adapted forms of *T. hyalipennis* (Fig. 3C), albeit not always consistently, as mentioned above. OPA2 could discriminate between the four main species investigated, but not the host-adapted forms of *T. hyalipennis*. Within species, it revealed no intraspecific differences.

Table 2 shows the genetic distances calculated between 'species' pairs from the RAPD data obtained, whilst Figure 4 represents a cladogram for the *Tetramesa* species studied along with two multivariate representations – MDS of Euclidean distances and factor analysis. Both the cladogram and MDS plot were constructed using the genetic distance data (see also Al-Barrak, 2001). Clearly, the cladogram shows *T. longicornis* to be the species most basal and most related to *T. hyalipennis s.l.*, whilst *T. calamagrostidis* and *T. petiolata* are more distantly related to these two species, yet at the same time most closely related to each other. The positioning of the node shows the

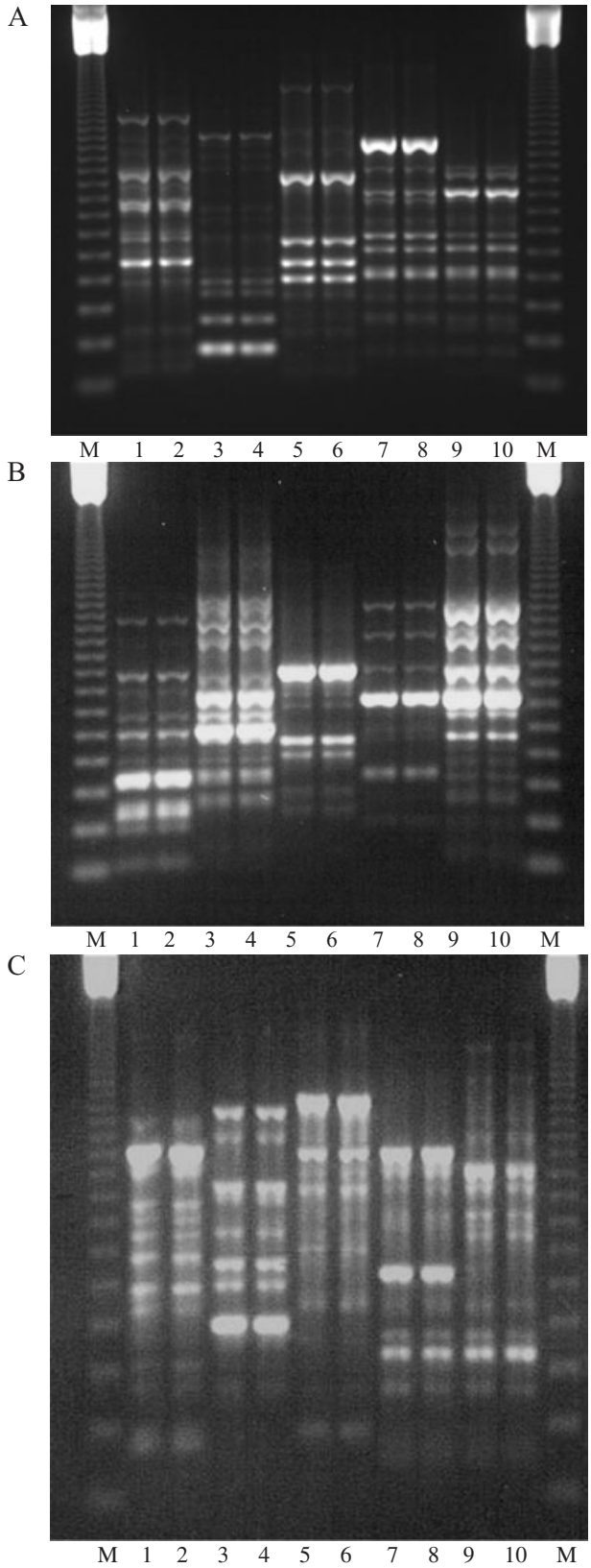

Figure 3. Electrophoretic banding patterns produced by primers OPG19 (A), OPF4 (B) and OPM15 (C). Lanes 1,2, *T. calamagrostidis*; 3,4, *T. longicornis*; 5,6, *T. petiolata*; 7,8, *T. hyalipennis* (ex: *E. repens*); 9,10, *T. hyalipennis* (ex: *E. farctus*); M, 123-bp molecular size ladder. The anode is at the bottom of the gel.

Table 2. Genetic distances between *Tetramesa* species (used for the cladogram and multidimensional scaling plot; Fig. 4A, B)

	T. calamagrostidis	*T. longicornis*	*T. petiolata*	*T. hyalipennis 1*	*T. hyalipennis 2*
T. calamagrostidis	0	17.46	16.40	18.44	16.82
T. longicornis	–	0	18.87	19.88	17.78
T. petiolata	–	–	0	19.62	18.06
T. hyalipennis 1	–	–	–	0	10.34
T. hyalipennis 2	–	–	–	–	0

T. hyalipennis 1 – *T. hyalipennis* (ex: *E. repens*).
T. hyalipennis 2 – *T. hyalipennis* (ex: *E. farctus*).

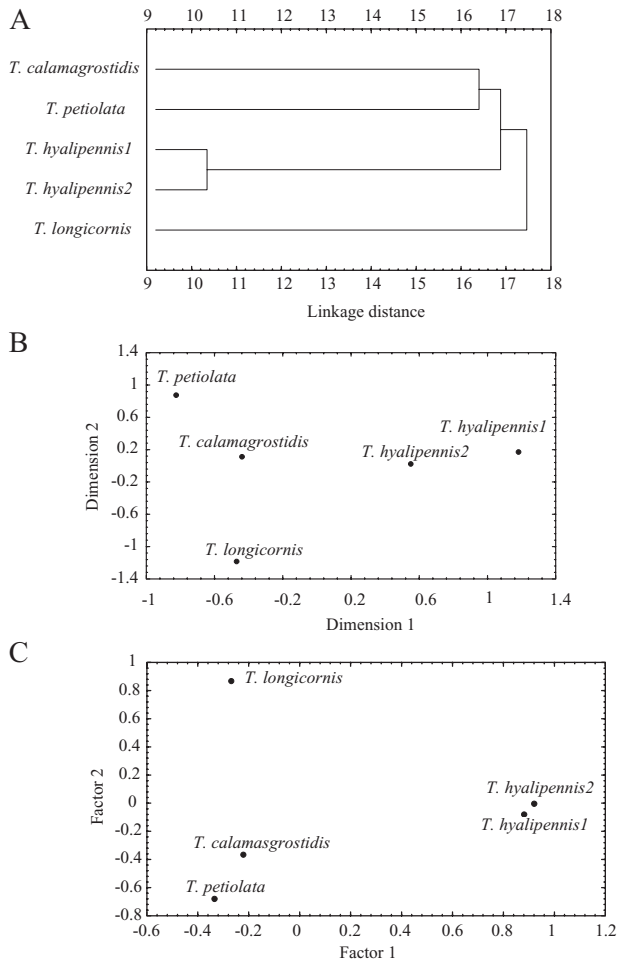

Figure 4. A, cladogram for five species of the genus *Tetramesa*. Distance measure: Euclidean; Linkage rule: Single (nearest neighbours). B, multidimensional scaling of Euclidean distances (stress = 0.000). C, factor analysis of RAPD data (Rotation: Unrotated; Extraction: Principal components).

two forms of *T. hyalipennis s.l.* to be most closely related of any of the samples investigated, and as such, these may have diverged genetically the most recently. MDS of Euclidean distances and factor analysis broadly confirmed these trends, especially the latter analysis (Fig. 4C).

DISCUSSION

For this study, enzyme electrophoresis was first used, followed by analysis of the same species using RAPD-PCR markers. RAPDs are particularly suitable for studies of small to minute organisms such as insects, from which only nanogram quantities of DNA can be extracted (Black *et al.*, 1992; Black, 1993). They also often show enhanced sensitivity in taxonomic discriminations compared with enzymes. Because of the advantages and sensitivity of the RAPD-PCR technique, it appeared to be the best marker system for our investigation, especially to clarify the status of *T. hyalipennis* (ex: *E. repens* and *E. farctus*) in relation to the other species tested. Both kinds of marker have already proved to be very useful in the recognition of very closely related species populations (see Loxdale, 1994 and Loxdale & Lushai, 1998, 2001 for overviews), including species belonging to the order Hymenoptera (e.g. Halliday, 1981; Hung, 1982; Castañera, Loxdale & Nowak, 1983; Pungerl, 1986; Dawah, 1987; Hung and Schaefer, 1990; Walton, Loxdale & Allen-Williams, 1990; Vanlerberghe-Masutti, 1994), especially species complexes and species boundaries, of which the group has many examples.

In addition to taxonomic discrimination, another important aspect of this kind of study performed in the absence of clear morphological criteria is what defines a 'true species' in terms of molecular genetic differences (see for example, Kunz, 2002), along with consideration of the ecological forces at work allowing divergence of electrophoretically distinguishable forms (Claridge, Dawah & Wilson, 1997a). By revealing such forms, ranging along a scale from ethospecies

to ecotypes, biotypes, races, and cryptic and sibling species, ideas of biodiversity within given ecosystems may need to be revised (see, for example, Eastop, 1973; Avise, 1977; White, 1978; Claridge, Dawah & Wilson, 1997a,b; Thomas & Singer, 1998 for definitions and discussion of these evolved forms). Clearly, in the case of sympatric populations, it is pertinent to determine what barriers exist – ecological, morphological, behavioural and genetic – that cause populations to diverge and ultimately, to differentiate and remain differentiated. Whilst much debate continues to surround the topic of sympatric speciation, for insects very few documented examples exist, including tephritid fruit flies, *Rhagoletis pomonella* (Walsh) (Feder, Berlocher & Opp, 1998), pea aphids, *Acyrthosiphon pisum* (Harris) (Via, Bouck & Skillman, 2000), hymenopterous parasitic wasps, *Aphidius ervi s.l.* (Atanassova *et al*., 1998) and tortricid larch budworm moths, *Zeiraphera diniana* Guenée (Emelianov, Mallet & Baltenweiler, 1995), and even their existence is controversial (see Via, 2001; Berlocher & Feder, 2002). We pose the question: do *Tetramesa* sp. offer further examples of sympatric speciation in insects, specifically the cryptic, host-associated forms of *T. hyalipennis*? Before discussing this, we briefly mention certain technical aspects relating to the electrophoretic discrimination of parasitic Hymenoptera.

It is often quite difficult to decide whether the enzyme banding patterns observed for parasitic Hymenoptera represent different alleles at one locus or different loci, a situation which cannot be resolved without formal crossing experiments. Additional complications in interpreting banding profiles may arise due to such factors as post-transitional modification of the enzymes concerned (Cameron, Powell & Loxdale, 1984). Since the insects used here were not crossed genetically, the banding patterns were recorded and utilized in a purely diagnostic way.

Electrophoresis does not allow discrimination of all variables. Electromorphs of an enzyme gene may, as a result of mutation, have a different amino acid sequence leading to a difference in size and hence electrophoretic mobility even though they have the same or similar charge. Alternatively, electromorphs may have a different shape due to an amino acid difference leading to a shape (protein folding) alteration that affects net charge and mobility, even though molecular weight is not significantly changed (see Richardson *et al.*, 1986 for details). Another difficulty with enzyme electrophoresis is that only structural genes coding for soluble proteins can be scored; regulatory genes or those coding for non-soluble proteins may behave differently and cannot be detected (Loxdale, 1994; Loxdale & Lushai, 1998). The lack of detectable electrophoretic differences between the two host forms of *T. hyalipennis*, as found here and also by Dawah (1987), could be due to the number of enzymes tested being too few. For example, Singh & Rhomberg (1987) showed that in closely related *Drosophila* species (*D. melanogaster* Meigen and *D. simulans* Sturtevant), some genetic species discriminations were possible using < 30 enzyme loci. However, results on a larger sample of loci from the same species revealed that complete divergence was possible even at a small number of diagnostic enzyme loci (7%) once these had been identified (Singh, 1989). Therefore, more enzyme loci undoubtedly need to be identified and examined before any definite conclusions can be drawn on the status of *T. hyalipennis* using enzymes.

The use of RAPDs provided a clearer picture of the taxonomic relationships existing between the *Tetramesa* species studied. The results were broadly consistent with similar inter- and intraspecific variations found in other insects using RAPDs in which differentiation of very closely related species or even geographical populations have been clarified, for example: Lepidoptera – Indian meal moth, *Plodia interpunctella* (Hübner) (Dowdy & McGaughey, 1996), European corn borer, *Ostrinia nubilalis* (Hübner) (Pornkulwat *et al*., 1998), gypsy moth, *Lymantria dispar* (L.) (Garner & Slavicek, 1996), Hymenoptera – *Trichogramma* spp. and *Anaphes* spp. (Landry, Dextraze & Boivin, 1993; Vanlerberghe-Masutti, 1994); Diptera – *Anopheles* spp. (Wilkerson *et al*., 1993; Favia, Dimopoulos & Louis, 1994), sand fly, *Lutzomyia migonei* Franca (Adamson *et al*., 1993); Homoptera – whitefly, *Bemisia tabaci* (Gennadius) (Gawel & Bartlett, 1993), Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Puterka *et al*., 1993), and the cotton-melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (Vanlerberghe-Masutti & Chavigny, 1998).

RAPD-PCR successfully discriminated the closely related sibling species of *T. calamagrostidis, T. longicornis* and *T. petiolata* (Fig. 4). The within and between species diversity observed may perhaps have occurred during the arrhenotokous parthenogenetic phase of the life cycle of each species (i.e. the production by unmated female wasps of unfertilized haploid males), as a result of the increased reproductive rate and the potentially enhanced host association in this phase, i.e. selection for host-adapted genotypes (see below). Probably, then, we are witnessing sympatric speciation at a local geographical level (Mopper & Strauss, 1998), which presumably would tend to break down during sexual recombination of the adults, since some species [*T. hyalipennes* in this study and *T. eximia* (Dawah, 1987)], do not appear to show behavioural barriers to inbreeding and males mate with females from their own host population as well as with those from the other host population tested (i.e. ex: *E. repens* or ex: *E. farctus* and *vice versa* in the case of *T. hyalipennes*). In these previous trials, mating

combinations were confirmed as being successful by the presence of sperm in the female spermatheca following dissection (see Dawah, 1987 for details). Nevertheless, our RAPD results do not provide strong evidence for the specific rank status of 'sibling species' for the two *T. hyalipennis* populations from different hosts. Thus there is no significant discontinuous genetic variation between these populations, neither of which is clearly reproductively isolated in space and time (Fig. 4A). This being so, the results do not overturn the earlier notion that the two forms represent the same species (*s.l.*, at any rate), as earlier proposed by Claridge (1961) and Dawah (1987). Yet there is clearly some degree of genetic divergence between the forms to the point where discrimination is possible between them.

As mentioned above in reference to the main *Tetramesa* species studied, an explanation for the observed genetic divergence between the two forms of *T. hyalipennis* is that they represent a single oligophagous population, but that the observed differences are the result of differential selection on the two hosts (M. F. Claridge, pers. comm.). However, again, being panmictic, it is then difficult to imagine how evolved host preferences could be maintained. Preference for natal host plant odours and assortative mating due to host-specific sex pheromones may well drive host specialization (Linn *et al*., 2003; Thomas *et al*., 2003), but the usual arguments arise as to how this is maintained in the face of reciprocal gene flow, as with the much studied *Rhagoletis pomonella* complex. In *R. pomonella*, according to Feder and coworkers, 'it is the interaction of host phenology, local ambient temperature conditions, and fly development that is responsible for this postzygotic selection', i.e. host specialization by apple and hawthorn-feeding forms (see Feder *et al*., 1998 and Berlocher & Feder, 2002 for further details and arguments concerning *R. pomonella*, and Thomas *et al*., 2003 for the mechanisms of sympatric speciation in *O. nubilalis*). In *R. pomonella*, recent evidence suggests that chromosomal inversion polymorphisms are involved in the maintenance of sympatric forms on their respective plant host, such that 'genes affecting diapause traits [that are] involved in host race formation reside within large complexes of rearranged genes' (Feder *et al*., 2003a). Feder *et al*. (2003b) also discuss evidence that allopatric speciation events, and involving inversion polymorphisms affecting key diapause traits and aiding North American flies to adapt to a variety of plants with different fruiting times, may have been initially involved in providing the 'important raw genetic material' facilitating adaptive radiation and hosts shifts in this fly. Ortiz-Barrientos *et al*. (2002), in a recent review, likewise discuss the genetic mechanisms, including chromosomal rearrangements, that may cause speciation in *Drosophila* in the face of reciprocal gene flow between natural populations.

Perhaps the chromosomal haplo-diploidy of Hymenoptera in which the male is haploid and bears a single gene at any locus under consideration not only often drives species populations towards homozygosity (because any negative selective trait is more greatly exposed to selection in the haploid condition; Menken, 1991), but it may also reinforce host preference by selection against 'incompatible' gene arrangements, for example, those that have not been the product of an adaptive chromosomal inversion to a particular plant host. If this were so, it would be broadly analogous to 'Haldane's Law' in which the heterogametic sex is selected against during hybridization of different species (Stebbins, 1958).

Opponents of the sympatric model of speciation, notably Mayr (1963), insist that species require a high degree of spatial isolation and of host or habitat choice (see Via, 2001). The implications of the existence of sibling species in many taxa currently assigned to nominal species name are enormous (Claridge *et al.*, 1997a). At present, because of the increasing global threat posed by human activity to habitats and associated wildlife, attempts are underway to map the total biodiversity of the planet (Gaston, 2000; Purvis & Hector, 2000; Dolphin & Quicke, 2001). The inclusion of sibling species would greatly increase the number of recognized species by many thousands or even millions (e.g. Lawton *et al*., 1998). Investigations into cryptic species are vital for pest management strategies (integrated pest management, IPM) and conservation policies alike. The ability to recognize and identify biological species (Claridge, 2003) with a unique genetic heritage is a fundamental necessity (Lawton *et al*., 1998; Basset, 2001).

These results emphasize that the application of molecular diagnostic approaches may well lead to a better understanding and greater reliability in evaluating the taxonomic status of closely related species of Hymenoptera. This advance would be particularly important for those species (e.g. parasitoids) used as biocontrol agents in IPM programmes to control insect pests. In addition, by confirming empirically, using genetic markers, the existence of some degree of host divergence occurring below the species level, as earlier noted for *R. pomonella* and aphids of various species (Via, 1999, 2001; Haack *et al*., 2000; Via *et al*., 2000; Lushai, Markovitch & Loxdale, 2002), our results further emphasize the fact that 'apparent' species populations occurring in the same geographical area are not necessarily homogeneous genetic entities. Rather, they may consist of numerous levels of evolutionary divergence ranging from geographical populations to truly genetically distinct species (Avise, 1977, 1994, 2000). The morphologically similar, host-adapted

forms of *Tetramesa*, especially *T. hyalipennis s.l.*, may indeed be a further example of sympatric speciation 'in action' (certainly to the level of host-adapted races or biotypes) and they may represent a suitable group of organisms to rival the aforementioned examples in studies of sympatric speciation in insects. If *T. hyalipennis* really is undergoing sympatric divergence, then it begs the question: what are the mechanisms – ecological and genetic – that are reinforcing, maintaining or extending this? Further morphometric and genetic studies using more sophisticated molecular markers such as mitochondrial DNA markers, microsatellites, amplified fragment length polymorphic markers (AFLPs) (e.g. Emelianov, Marec & Mallet, 2004 in the case of the sympatric host races of *Z. diniana*) and, ultimately, DNA sequencing of mitochondrial and ribosomal DNA regions, in association with chromosomal and behavioural investigations (e.g. host plant preference, mate-choice experiments, phenological studies, pheromone differentiation and acoustic signals; Claridge, 1991), will doubtless, as with *Rhagoletis*, further advance our understanding of the ecological and evolutionary processes involved.

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APPENDIX

Evaluation of RAPD-PCR fingerprints generated from *Tetramesa* species with 55 primers

No.	Primer name	Sequence	No. of polymorphic bands		
			Total	Well-amplified	Faint
1	OPA2*	5′TGCCGAGCTG-3′	19	11	8
2	OPA3*	5′AGTCAGCCAC-3′	30	11	19
3	OPA5*†	5′AGGGGTCTTC-3′	33	12	21
4	OPA7	5′GAAACGGGTG-3′	0	0	0
5	UB1*	5′CCTGGGCTTC-3′	34	14	20
6	UB2*†	5′CTTGGGCTTG-3′	26	10	16
7	UB58*	5′TTCCCGGAGC-3′	27	7	20
8	UB62	5′TTCCCCGTCG-3′	0	0	0
9	OPG16*	5′AGCGTCCTCC-3′	29	16	13
10	OPG19*†	5′GTCAGGGCAA-3′	24	14	10
11	OPE6	5′AAGACCCCTC-3′	0	0	0
12	OPE19*	5′ACGGCGTATG-3′	26	14	12
13	OPI13*	5′CTGGGGCTGA-3′	26	20	6
14	OPK4	5′CCGCCCAAA-3′	0	0	0
15	OPK16*	5′GAGCGTCGAA-3′	25	11	14
16	OPF1*	5′ACGGATCCTG-3′	35	11	24
17	OPF2*	5′GAGGATCCCT-3′	34	14	20
18	OPF3*†	5′CCTGATCACC-3′	34	7	27
19	OPF4*†	5′GGTGATCAGG-3′	25	9	19
20	OPF5	5′CCGAATTCCC-3′	0	0	0
21	OPF6	5′GGGAATTCGG-3′	0	0	0
22	OPF7*†	5′CCGATATCCC-3′	16	7	9
23	OPF8	5′GGGATATCGG-3′	2	0	2
24	OPF9	5′CCAAGCTTCC-3′	10	0	10
25	OPF10	5′GGAAGCTTGG-3′	29	18	11
26	OPF11*	5′TTGGTACCCC-3′	22	6	16
27	OPF12	5′ACGGTACCAG-3′	7	4	3
28	OPF13*	5′GGCTGCAGAA-3′	31	22	9
29	OPF14	5′TGCTGCAGGT-3′	0	0	0
30	OPF15	5′CCAGTACTCC-3′	0	0	0
31	OPF16	5′GGAGTACTGG-3′	0	0	0
32	OPF17	5′AACCCGGGAA-3′	0	0	0
33	OPF18	5′TTCCCGGGTT-3′	0	0	0
34	OPF19	5′CCTCTAGACC-3′	0	0	0
35	OPF20	5′GGTCTAGAGG-3′	0	0	0
36	OPM1*†	5′GTTGGTGGCT-3′	18	7	11
37	OPM2*	5′ACAACGCCTC-3′	18	5	13
38	OPM3*	5′GGGGGATGAG-3′	18	6	12
39	OPM4*	5′GGCGGTTGTC-3′	26	12	14
40	OPM5*	5′GGGAACGTGT-3′	21	4	17
41	OPM6	5′CTGGGCAACT-3′	23	10	13
42	OPM7*	5′CCGTGACTCA-3′	22	12	10
43	OPM8*	5′TCTGTTCCCC-3′	22	12	10
44	OPM9*	5′GTCTTGCGGA-3′	22	9	13

APPENDIX *Continued*

No.	Primer name	Sequence	No. of polymorphic bands		
			Total	Well-amplified	Faint
45	OPM10*	5′TCTGGCGCAC-3′	28	15	13
46	OPM11*	5′GTCCACTGTG-3′	29	13	16
47	OPM12*	5′GGGACGTTGG-3′	21	11	10
48	OPM13*	5′GGTGGTCAAG-3′	14	5	9
49	OPM14*	5′AGGGTCGTTC-3′	25	14	11
50	OPM15*†	5′GACCTACCAC-3′	14	5	9
51	OPM16*	5′GTAACCAGCC-3′	29	12	17
52	OPM17*	5′TCAGTCCGGG-3′	14	5	9
53	OPM18*	5′CACCATCCGT-3′	24	16	8
54	OPM19*	5′CCTTCAGGCA-3′	8	7	1
55	OPM20*	5′AGGTCTTGGG-3′	23	16	7

*Primers ($N = 37$) gave clear and consistent banding pattern differences between the five main species.
†Primers ($N = 8$) produced variable results for the two host-associated populations of *T. hyalipennis*.