sex chromosome (X) in all *Hemideina* species has been identified as one of the large metacentric chromosomes (Morgan-Richards 1997) that always occur in odd numbers in males, but are paired in females. However, as all tree two karyotypes have more than three large metacentric chromosomes of similar size, the exact identity of the sex chromosome is uncertain.

Hemideina crassidens (Blanchard) (Fig. 1) has two known chromosome races, one with a diploid (2n) number of 15 (XO males)/16 (XX females) and one race with 19/20 chromosomes. The difference in diploid number between the sexes is due to an XO sex determination system; but the variation in chromosome number that differentiates the two races is the result of the fusion/fission duce F_1 offspring in laboratory crosses (Morgan-Richards 2000). *Hemideina thoracica* (White) has nine known chromosome races, which have karyotypes with between 12 and 24 chromosomes (female 2n). The chromosome races of *H. thoracica* interbreed in narrow regions of contact and limited introgression of mitochondrial and nuclear alleles has been shown (Morgan-Richards 1997; Morgan-Richards et al. 2000, 2003; Morgan-Richards et al. 2001b). This suggests that chromosomal differences alone are not sufficient to cause reproductive isolation between chromosome races in this genus.

The seven species of *Hemideina* have broadly parapatric ranges, with narrow regions of overlap. Hybridisation is possible between some *Hemideina* species (Morgan-Richards et al. 2001a) although all evidence is of F_1 hybrids only based on morphology of wild specimens or captive observations. As yet, it is unknown if viable F_2 hybrids are produced leading to introgression of alleles between *Hemideina* species. In one species pair (*Hemideina ricta* and *Hemideina femorata*) the production of F_1 hybrids has not led to genetic introgression (Morgan-Richards & Townsend 1995).

The present study examines the three parapatric species of *Hemideina* in the North Island of New Zealand, all of which come into contact at the borders of their range. The 17/18 chromosome race of *H. thoracica* occurs in the southern part of the North Island of New Zealand where it overlaps with the 15/16 chromosome race of *H. crassidens* in the Manawatu region (Fig. 1). The 17/18 chromosome race of *H. thoracica* also occurs in Hawkes Bay where it overlaps with *Hemideina trewicki* (Morgan-Richards), which has only one known chromosome race (2n = 17/18, Fig. 1). Putative F_1 hybrids have been identified based on body colouration and karyotype in Hawkes Bay (Morgan-Richards et al. 1995), but quantitative analyses of chromosomes are required to confirm these identifications, and to investigate whether F_1 hybrids are fertile.

We document karyotype variation for the three w t species at the regions where *H. thoracica* and *H. crassidens* overlap in the Manawatu, and *H. thoracica* and *H. trewicki* overlap in Hawkes Bay. To increase karyotype resolution the analysis uses chromosome measurements to describe both relative chromosome length, and centromeric position based on arm-length ratios. This quantitative approach to karyotype characterisation is important for studying chromosomal variation within species and comparing among them. This tool will help identification of chromosomes that are unique to each species and so provide a robust method for detection of F_1 hybrids and possible backcross individuals.

Methods

Ten specimens each of *H. thoracica* and *H. crassidens* were collected from Manawatu, where they are sympatric (Kahuterawa Valley GPS: 40.47184S, 175.60943E, Fig. 1). Nine *H. thoracica* and thirteen *H. trewicki* were collected from Hawkes Bay (Mohi Bush Scenic Reserve: 39.85S, 176.88333E and Elsthorpe Scenic Reserve: 39.91881S, 176.8042E, Fig. 1). Only males were used for this study as chromosome numbers differ between sexes due to their XO sex determination system, and males provide tissue with high mitotic rates. Species were identified using their unique colour combinations of pronotum and abdomen (Ramsay & Bigelow 1978). For each w t cauor20(o)9(i)13.91(ra)24..6(n)-6.5(o)-325.1(27 metaphase or anaphase chromosome spreads at 1200× magnification using an Olympus BX51 Compound Microscope. CellSense Dimension 1.6 software (Olympus) was used to measure chromosomes. Each chromosome arm was measured separately and combined to give total chromosome length. For each mitotic spread measured, individual chromosomes were then ranked and numbered from longest to shortest. This information provided data for arm-length ratios for the nomenclature developed by Levan et al. (1964). Because chromosome contraction is expected to result in variation among cells at different stages of cell division, the ratio of each chromosome to the total chromosome material per cell spread was calculated to allow comparison of relative chromosome sizes among cells and among individual w t . Diploid species are expected to have pairs of homologous chromosomes that do not differ from one another significantly in length. A two-sample *t*-test implemented in Minitab 16 Statistical Software (Minitab Inc.) was used to compare the ranked individual chromosome lengths to identify likely chromosome pairings. This method allowed for inferences to be drawn about possible pairs by comparing the *P*-values of neighbouring chromosome pairs, although this method would only allow comparisons with a relatively small data set, as was used here. With larger samples, the *P*-values would decrease to zero and all chromosomes would be significantly differentiated because of being ranked by size before the *t*-tests were performed. As the pairs and sex chromosomes have only putatively been identified using visual methods in the past (e.g. Morgan-Richards 1997), we developed this approach to provide an unbiased method for identification of the unpaired sex chromosome.

Results

The karyotypes from the two individual w t from a single population (*H. thoracica* from Hawkes Bay) were compared. Each chromosome in 10 mitotic cells was measured and variation between individuals was assessed visually using p-arm by q-arm scatter plots of all chromosomes (Fig. 2A). Almost complete overlap was observed for the range of chromosome shape and relative size in the two w t karyotypes. Only one pair of their smaller chromosomes can be visually identified on the graph (Chromosome 10 and 11, Table 1). The autosomes can be divided into a cluster of larger metacentric chromosomes and a cluster of small pairs. Two of the smaller chromosomes (10th and 11th) have two arms but the smaller arm (p-arm) is only visible (and measurable) when mitosis has proceeded to only partial chromosome contraction. Full chromosome contraction results in mitotic cells where these chromosomes appear to have a single arm. Hence, for both individual w t, two clusters of these chromosomes are resolved on the graph resulting from variation in mitotic stage and hence visibility or not of the p-arm. After confirming that individual cells from the same population have similar karyotypes, one mitotic spread from each specimen ($n = 9$, $n = 10$, $n = 10$, $n = 13$) from each of the four populations was measured and analysed. The w t from the two *H. thoracica* populations have very similar karyotypes (Tables 1, 2, Fig. 2B). Both have nine large metacentric chromosomes that vary in relative size by only a small amount. Two smaller submetacentric chromosomes (10 and 11) were identified in the Hawkes Bay group of *H. thoracica*, although as the centromeres were only visible in about half of the spreads measured, it is likely that centromeres were not visible in the Manawatu group because of greater chromosome constriction, as they have a similar total length and are probably homologous. The last six chromosomes in each of the two *H. thoracica* populations were very small, and no centromere could be identified. They were labelled telocentric, and the ratio was given based on an arbitrary value of 0.1% for the p-arm. Chromosome nine was significantly differentiated in the Hawkes Bay sample and in the first individual w t studied. For the Manawatu *H. thoracica* and the second individual w t , n.2(ia)1316.1M14.6(w)]T7(be)-252.3

Table 1 Average relative length of each chromosome in the karyotype of Hawkes Bay male *Hemideina thoracica* (n = 10), and the average ratio for arm lengths (q/p) provides centromere position, and nomenclature based on et al. (1964). Metacentric (m 1.05–1.66); submetacentric (sm 1.67–2.99); subtelocentric (st 3.00–6.99); telocentric (t 7.00–39.00). The *P*-value used to assign chromosome pairs was taken from two-sample *t*-tests for differences between chromosomes.

Chromosome	Relative length ± SD	Ratio (q arm/p arm)	Type	Putative pair	P-value
1	10.98 ± 0.82	1.27	m		
				1	0.288
2	10.55 ± 0.82	1.28	m		
					0.051
3	9.88 ± 0.41	1.46	m		
				2	0.304
4	9.69 ± 0.37	1.27	m		
					0.008
5	9.23 ± 0.21	1.53	m		
				3	0.386
6	9.11 ± 0.37	1.38	m		
					0.027
7	8.68 ± 0.36	1.38	m		
				4	0.019
8	8.27 ± 0.3	1.36	m		
					0.006
9	7.71 ± 0.52	1.45	m	Sex Chr	
					<0.001
10	2.88 ± 0.31	5.12	sm		
				5	0.025
11	2.5 ± 0.34	5.33	sm		
					0.173
12	2.2 ± 0.54	24.83	t		
				6	0.284
13	1.95 ± 0.37	21.8	t		
					0.162
14	1.72 ± 0.3	19.16	t		
				7	0.777
15	1.67 ± 0.44	17.17	t		
					0.548
16	1.56 ± 0.28	17.3.777			

When individual chromosomes were compared statistically using a two-sample *t*-test (Table 3), the fifth chromosome was shown to be most significantly differentiated from its two neighbouring chromosomes, and is therefore most likely to be the sex chromosome (Fig. 3C).

Hemideina trewicki also has a distinct karyotype, with only seven large metacentric chromosomes, that roughly matched the relative size of the largest chromosomes from *H. thoracica* and *H. crassidens* (Figs 2D–2E; Table 4). There appeared to be four pairs of chromosomes that all roughly correspond to the size and centromeric position of the intermediate-sized (second-smallest) chromosome pair of *H. crassidens*. The smallest pair matches the smallest chromosomes from both *H. crassidens* and *H. thoracica*. The sixth and seventh chromosomes appeared to make a pair when examined visually, and the two-sample *t*-test values support this inference; however, as these chromosomes overlap in size with the larger *H. thoracica* and *H. crassidens* chromosomes they are not distinguishable among species. Overall, despite differing

numbers of chromosomes, *H. crassidens* and *H. tre-welcki* appear to have eree sha-
with oms zin have

Discussion

The two populations of *H. thoracica* from Hawkes Bay and Manawatu belong to the same chromosome race, and all three species examined here have distinct karyotypes. The *H. thoracica* karyotype has the only autosome pair that is well-differentiated from all other chromosomes in the complement (chromosome 10 and 11; pair 5). *Hemideina crassidens* and *H. trewicki* have chromosomes that are not shared with *H. thoracica*. Both *H. thoracica* and *H. crassidens* have unique chromosomes that can be used to distinguish them as a species/chromosome race. *Hemideina thoracica* and *H. trewicki* also have quantitative differences in the number of certain sized chromosomes (three very small pairs in *H. thoracica* as opposed to one in both other species, and four intermediate sized pairs between 3.11 and 4.74 units (see ratio column in Tables 3 and 4) in *H. trewicki* as opposed to one in *H. crassidens* and none in *H. thoracica*). These differences may be important in distinguishing the ancestry of chromosomes in the karyotype of putative hybrids.

Previous cytogenetic studies that aimed to compare hybrid zones in *Hemideina* spp. have labelled the longest chromosome in the karyotype as the sex chromosome (e.g. Morgan-Richards 1997). As all chromosomes are paired in males except the sex chromosome, the odd number of large chromosomes in each karyotype implies that one is unpaired and therefore the sex chromosome. However, as all chromosomes vary only slightly in size it was impossible to accurately visually identify one solitary chromosome that did not match any other in most *Hemideina* karyotypes. It is possible that the sex chromosome is identical in size to one of the autosomal pairs, although the results of the detailed measurements and comparison of relative chromosome length after ranking revealed that for *H. crassidens*, the fifth chromosome is most