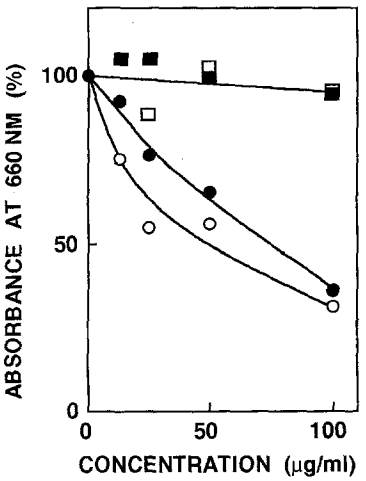

Figure 4. Antibacterial activity of halocyamine A against marine bacteria. The absorbance at 660 nm in the absence of halocyamine A in each bacterium was defined as 100%. *A. aquamarinus* (○), *P. perfectomarinus* (●), *A. putrefaciens* (□), and *V. anguillarum* (■).

cyamine B showed antibacterial spectra similar to those of A (not shown). The reasons for lack of antimicrobial activity of halocyamines against the latter two bacteria remain unknown.

Antimicrobial substances[9] and lectins[10,11] have been proposed as humoral factors in the defense mechanisms of invertebrates. In ascidians, lectins have been proposed as recognition molecules for foreign substances[12]. With respect to antibacterial substances in ascidians, we have already proposed that halocyamines present in hemocytes may have an extracellular function in the defense mechanisms of *H. roretzi*[5]. It has been demonstrated that *H. roretzi* hemocytes undergo several cellular responses such as phagocytosis[13] and lysis (contact reaction[14]) against foreign (non-self) substances.

The results described in this paper showing that halocyamines have an inhibitory effect on the growth of two kinds of fish disease viruses and two kinds of marine bacteria, support the above assumption that halocyamines could function as defense agents against viruses and/or bacteria invading the hemolymph of *H. roretzi*. The antiviral and antimicrobial activities of the halocyamines described in this study were lower than those of antiviral and antimicrobial substances isolated from colonial ascidians[1-3] by approximately two orders of magnitude. In this connection, we have shown that the combined concentrations of the two halocyamines accumulated in only one type of hemocyte, a vesicular cell[13], can reach a level as high as 10 mg/ml[5]. This type of cell was the most abundant (more than 50%) hemocyte in the hemolymph of *H. roretzi*. Therefore, the total concentrations of halocyamines could well be high enough to attack viruses and bacteria invading the hemolymph.

In preliminary studies, we found that halocyamine A showed acute toxicity against mice at a concentration of more than 100 mg/kg. This fact raises a hope that halocyamine and its derivatives might be candidates for use as medicines applicable to some human diseases.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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0014-4754/90/101066-03$1.50 + 0.20/0

Mitochondrial DNA variation in social wasps (Hymenoptera, Vespidae)

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Received 23 February 1990; accepted 26 June 1990

Summary. Patterns of restriction fragment length polymorphisms (RFLP) of European Vespinae were more similar within genera than between them. Distance trees were constructed that support the hypothesis of monophyly of the genera *Vespula* and *Dolichovespula*. Within the genus *Vespula*, *V. germanica* was more closely related to *V. rufa* than to *V. vulgaris*. The position of the genus *Vespa* remained uncertain due to the precision limits of the RFLP technique.
Key words. Vespinae; mitochondrial DNA; restriction fragment length polymorphism (RFLP).

Linnaeus [1] classified the social wasps in one genus which he called *Vespa*. Since then many authors have dealt with the problem of classification and phylogeny of the social wasps. Frequently the opinions about monophyly and the evolutionary steps of the individual genera differ substantially.

De Beaumont [2] suggested a Vespinae phylogeny based on morphological characters where the genera *Dolichovespula* and *Vespula* are two separate monophyletic groups. He divided the *Vespula* into *Paravespula* (*P. vulgaris* and *P. germanica*) and *Vespula* (*V. rufa* and *V. austriaca*). Yamane [3] compared morphological and behavioral larval characters. Sticking to the monophyly of *Vespula* and *Dolichovespula*, he suggested a phylogenetic tree with *Vespula* as the yellowjacket genus with the most primitive features, and *Dolichovespula* with more derived features. Varvio-Aho et al.[4] investigated the genetic relationship of eight northern European yellowjacket species in an isozyme study. In their tree, *D. media* shows a greater distance from *D. saxonica* than from *V. vulgaris*. *V. vulgaris* was only weakly related to *V. rufa*. Carpenter [5] constructed a phylogenetic tree based on seventeen morphological characters. This tree supports the monophyly of *Vespula* and *Dolichovespula*. The *Vespula* group is divided into *Paravespula* and *Vespula*, corresponding to De Beaumont's [2] system. Carpenter [5] obtained a phylogenetic sequence with *Vespa* as a sister-group of the remaining yellowjackets. A common basic assumption is that the genus *Vespa* has the most primitive features in both morphology and behavior [2, 3, 6]. This suggests that a *Vespa*-like ancestor forms the phylogenetic basis of the Vespinae. The monophyly of the subfamily Vespinae was strongly supported by Carpenter [7].

In this study we used restriction fragment length polymorphisms (RFLP) of Vespinae mitochondrial DNA (mtDNA) to obtain more information about phylogenetic relationships within the Vespinae.

The molecular basis of mtDNA polymorphism has been described by numerous authors [8–10]. MtDNA is a powerful tool for reconstructing the phylogenetic relationships between and within species [11].

Material and methods

Wasp nests were collected within an area of 50 km around Erlangen in southern Germany. We used three nests each of six wasp species of the three genera: *Vespula* (*V. vulgaris, V. germanica, V. rufa*), *Dolichovespula* (*D. saxonica, D. media*), and *Vespa* (*V. crabro*). From each nest about 1 g of larvae were taken and analyzed.

The intestine of each individual larva was excised and the tissue homogenized, using a method similar to that of Moritz et al.[12], in 0.25 M sucrose-TE-buffer (0.25 M sucrose, 0.03 M Tris, 0.01 M EDTA, pH 8) in a Potter-Elvehjem homogenizer with a teflon pestle. In a first centrifugation (700 × g, 4 °C for 20 min) cell debris and nuclear DNA was pelleted. The supernatant was centrifuged again at 11,000 × g, 4 °C for 20 min, to isolate the mitochondria.

The pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and treated with SDS (1% in final concentration) and proteinase K (50 µg/ml) for 2 h at 40 °C. Proteins were extracted with phenol and subsequently with chloroform and ether as described by Maniatis et al.[13].

Ether was removed by heating the sample to 60 °C for 15 min. 5 M NaCl was added to a final concentration of 0.1 M and the mtDNA precipitated in ethanol at −70 °C. The mtDNA was pelleted at 12,000 × g (30 min, 0 °C) and washed in 70% ethanol. The sample was dried under vacuum for 15 min and resuspended in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8).

We used 1–5 units of enzyme (BRL Gibco: Acc I, Bcl I, Bgl II, EcoR I, Hind III, Mbo I and Xba I,) for each restriction digest of about 50 ng mtDNA in a total suspension volume of 20 µl. The buffer and temperature conditions used were as recommended by the supplier. The fragments were end-labelled by adding 1 µCi α-^{32}P ATP, unlabelled deoxy-nucleotide triphosphates (dCTP, dGTP, dTTP to a final concentration of 0.5 mM), and 0.4 U Klenow-fragment (large fragment of DNA polymerase I). After incubation for 20 min at room temperature the reaction was terminated by adding EDTA to a final concentration of 20 mM.

The samples were stained with a color marker (0.08% bromophenol blue, 60% sucrose, 0.05 M EDTA) and loaded into a 1% submarine horizontal agarose gel. Electrophoresis was carried out with 50 mA/31 V for 13 h in TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8). The fragments were visualized after drying by autoradiography on Cronex 4 X-ray film for 3 h at −70 °C using DuPont Cronex Hi Plus intensifying screens.

Results

Two of the restriction patterns produced by Hind III, Mbo I and Bcl I, EcoR I are shown in figure 1 and figure 2. We used a bacteriophage lambda DNA digest as a size standard (EcoR I + Hind III). For further evaluation we used the restriction patterns produced by the 6-basepair enzymes Acc I, Bcl I, EcoR I, Hind III and Xba I. The fragments produced by these and other restriction endonucleases are shown in the table. There were no RFLPs between the three nests of the same species.

The genetic similarity between various pairs of organisms can be estimated by the portion of shared fragments (F-value) in their restriction pattern. This fragment method of Nei and Li [14] is based on the following equation:

$$F = \frac{2N_{xy}}{N_x + N_y} \quad (1)$$

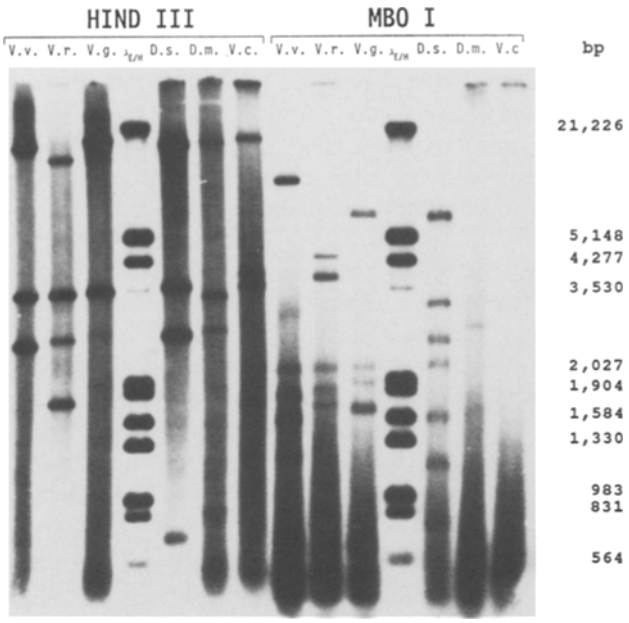

Figure 1. Hind III, Mbo I, digestion of mtDNA from social wasps. A lambda-EcoR I/Hind III digestion is used as length standard: 21,226--5,148-4,973-4,277-3,530-2,027-1,904-1,584-1,330-983-831 and 564 bp. V.v. = *Vespula vulgaris*, V.r. = *V. rufa*, V.g. = *V. germanica*, D.s. = *Dolichovespula saxonica*, D.m. = *D. media*, V.c. = *Vespa crabro*. Lanes 13 and 14 not interpretable.

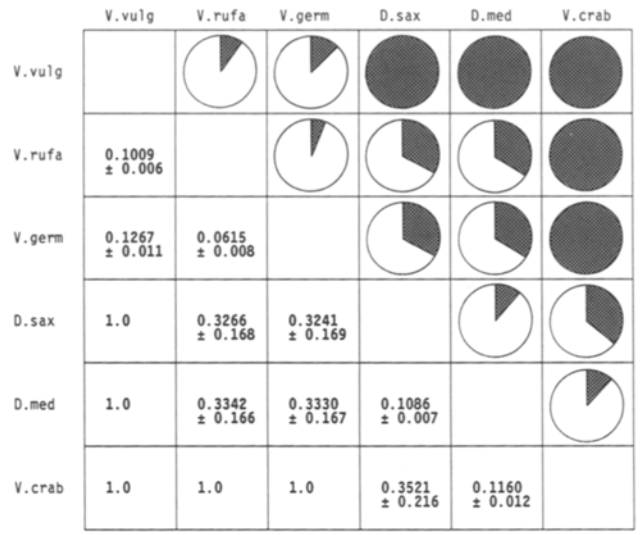

Figure 3. The genetic distance estimated by the equations given by Nei and Li[14] and Upholt[15] with standard error between pairs of species (lower half of the matrix). The same values represented as pie charts (upper half). Shaded area = distance.

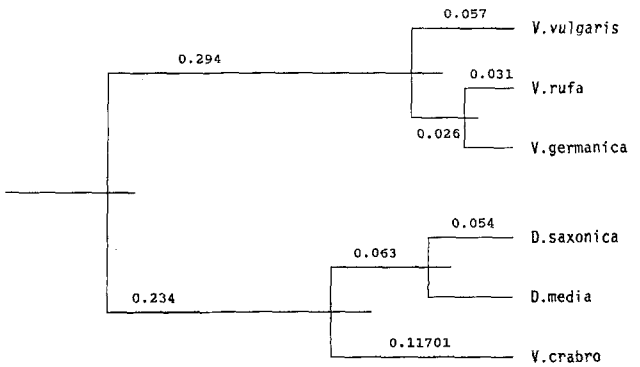

Figure 4. Distance tree based on RFLPs of six European social wasps, with the standard error produced by the UPGMA method. The values above the horizontal lines represent the genetic distances.

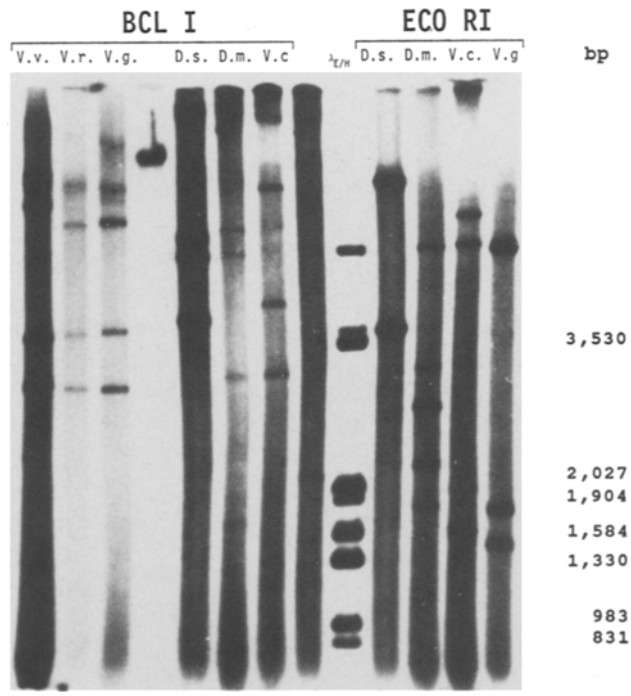

Figure 2. Bcl I, Eco RI digestion of mtDNA from social wasps. A lambda-EcoR I/Hind III digestion is used as length standard: 21,226-5,148-4,973-4,277-3,530-2,027-1,904-1,584-1,330-983-831 and 564 bp. Lanes 4 and 8 not interpretable. Species as in figure 1.

Where N_x and N_y are the numbers of fragments in organism X and Y. N_{xy} is the number of fragments shared between the two organisms.

The proportion of fragments shared between two organisms (F) can be used to estimate the average number of nucleotide substitutions per restriction site (δ) for each pair of organisms. We estimated the genetic distance (δ) using Upholt's[15] equation:

$$\delta = 1 - \left[\frac{-F + (F^2 + 8F)^{1/2}}{2}\right]^{1/r} \quad (2)$$

Where r is the number of base pairs recognized per cleavage site.

To estimate standard errors for the δ-values we used the jackknife procedure developed by Tukey[16], done over restriction enzymes (fig. 3).

The genetic distances were used to construct a phylogenetic tree. Because the distance matrix showed some violations of the triangle inequality, we used the UPGMA

Size (in base pairs) of mtDNA fragments obtained after digestion by restriction enzymes from the six European wasps. The arithmetic means ($\bar{X}$) of the mt genome size and standard errors ($s_{\bar{x}}$) are given below.

	V. vulgaris	*V. rufa*	*V. germanica*	*D. saxonica*	*D. media*	*V. crabro*
EcoR I	16788	6026	7413	17783	7810	10000
	1585	4266	4467	4677	3631	7810
		3631	1850		3020	1862
		1905	1738		2188	1400
		1585	1413		1738	
Hinf I				12589		
				2818		
				1585		
				977		
				832		
Hind III	14900	10000	16050	15382	15382	16503
	3548	3548	3548	4266	3508	4786
	2570	2630		2630	2700	4266
		1659		708	933	
Mbo I	8128	4786	7080	6310		
	2042	4074	7000	3467		
	1738	4000	2239	2692		
	1549	2239	1950	2291		
	1479	1862	1622	1549		
	1148	1698	631	1122		
	678			741		
Bcl I	13962	12589	12589	8318	8710	15031
	4786	4786	4786	7413	7413	6166
	3598	3548	3548	5129	3631	3631
					1641	
Acc I	14125	10000	10000	11220	16982	
	3891	7586	7073	6607	1349	
	2754		3090	1349		
Bgl II		12023				
		5888				
Xba I	17783	7943	14125	14125	9120	17370
	2344	5248	5248	5012	4842	1907
	1072	2455	1182	3981	3713	
					1109	
					692	
$\bar{X}$	20078	17996	19774	20796	20022	22683
$s_{\bar{x}}$	±850	±599	±607	±794	±836	±1501

method of Sokal and Michener[17,18]. With this method the principle of triangle inequalities is not required[18]. The resulting distance tree for the six social wasps analysed is shown in figure 4. Other parsimonious trees (Fitch-Magoliash, mixed Wagner-Sokal, PHYLIP 3.2 package, provided by Dr Felsenstein) yielded similar topologies.

Discussion

The RFLP method used in this study is a useful tool for estimating the relationship between species which are relatively closely related, i.e. between species belonging to the same genus. To estimate the relationship between species which are very divergent, i.e. *V. crabro* and *Vespula* species, the fragment method is not very accurate, and the restriction site method[14,18] estimates with a higher precision. Nevertheless, our data fit nicely into some of the previously published models of wasp phylogeny, though not into others.

The topology of the distance tree deduced from the distance matrix indicates a close relationship between *Vespa crabro* and the *Dolichovespula* group. This is mainly determined by the low genetic distance estimated between *V. crabro* and *D. media*. A more detailed comparison of mtDNA cleavage maps may be necessary to derive more exact estimates.

Furthermore, RFLPs in this study are mainly produced by 6-base enzymes which only yield large fragments. It is a problem to estimate the exact length of large fragments in agarose gels. Therefore our estimates of the total mt genome length suffer from large standard errors. By using restriction endonucleases with 4-base recognition sites the mt genome length could be estimated more exactly.

The phylogenetic tree supports the hypothesis of the monophyly of the genera *Vespula* and *Dolichovespula*. This is in agreement with studies of De Beaumont[2] and more recently Carpenter[5]. Varvio-Aho et al.[4] questioned the monophyly of *Vespula* and *Dolichovespula* by comparing isozyme polymorphisms. However, Carpenter[19] pointed out that the heterogeneity within the genera was the result of a misinterpretation of their isozyme data.

In contrast to models which divide the genus *Vespula* into *Paravespula* (*P. vulgaris* and *P. germanica*) and *Vespula*

(*V. rufa*)[2,5], we found that *V. germanica* was the closest to *V. rufa*. Yamane[3] concluded on the basis of larval characters that *Dolichovespula* species share some derived characters and are less closely related to *Vespa* than to *Vespula* species. In our study we found, in accordance with Green[20], that the ancestral genus of *Vespa* is more related to the genus of *Dolichovespula* than to the genus of *Vespula*. This is in agreement with the thesis that the *Vespula* group represents the most recently derived group.

The present study gives further evidence for the phylogenetic relationship of six European social wasps, based on genetic data. The results are largely in agreement with morphological studies[2,20], with the exception of dividing *Paravespula* and *Vespula* into distinct groups. The next step in the study of wasp mtDNA should be the analysis of restriction site maps, which will give us further details of Vespinae phylogeny.

Acknowledgments. This work was supported by the Deutsche Forschungsgemeinschaft (Grant No. Mo 373/4-1; RFAM).

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0014-4754/90/101068-05$1.50 + 0.20/0

Induction of chromosome aberrations and chlorophyll mutations in plants by methylisocyanate (MIC) gas

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Received 11 September 1989; accepted 13 March 1990

Summary. Seeds of *Solanum surattense* Burm. f. collected from areas of Bhopal (India) affected by methylisocyanate gas showed chromosome aberrations in root cells, and growth retardation and chlorophyll mutation of seedlings, the frequencies of which varied from one locality to another.
Key words. Solanum surattense; methylisocyanate gas; chromosome aberrations; chlorophyll mutants; interlocality variations.

Though hazardous in nature, the actual lethal potency of methylisocyanate (MIC) gas was not known before the fateful event on December[2-3], 1984, at the Union Carbide Factory at Bhopal. The venomous vapour killed more than 3000 people and numerous animals and plants[1,2]. Besides its lethal effects, it produced morphological lesions and cytogenetic abnormalities in living organisms[3-6]. In vivo study revealed that MIC and its reaction products cause mutagenicity in cultured mammalian cells[7]. It was shown to inhibit gametophyte morphogenesis and caused chlorophyll deficiency in fern gametophytes[4,8], and induced chromosome aberrations in pollen mother cells[5]. In order to understand the extent to which cytogenetic damage is carried into the progeny plants, MIC-exposed seeds were sown and the seedlings studied cytomorphologically.

Materials and methods

Wild *Solanum surattense* seeds were gathered from five areas known to be gas-affected and also from unaffected ones (control area)[5] in August, 1985. Five different sites in each area were randomly selected and from each site 10–15 ripe fruits of five different plants were collected and their seeds stored in a desiccator for a couple of months at room temperature. These were grown in the following November on sand beds. Seedling growth was measured on 15-day-old seedlings on a dry weight basis, and seedling survival was recorded three times a week until no more deaths occurred. For cytological observations germling root-tips were fixed in Carnoy's solution (acetic acid: alcohol, 1:3) overnight, and squashed in 1% acetoorcein. About 250 cells per sampling area were analysed for each kind of chromosome aberration.