

Comparison of the medium molecular weight venom fractions from five species of common social wasps by MALDI-TOF spectra profiling

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The average spectral profiles and the exact mass weight (MW) of biomolecules present in the medium fraction (from 900 to 3000 Da) of the venom of five social wasps (three European and one North American *Polistes* and the European hornet *Vespa crabro*) were determined by matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS. Data were obtained analyzing the venom of single specimens ($N = 46$) and elaborated with the ClinProTools 2.0 (CPT) software to search for differences among the five species examined. Interesting differences in the spectral profiles were found, allowing the discrimination of venoms belonging to the different species, and their possible use as a quality control method in venom immunotherapy (VIT) for allergic patients. Copyright © 2006 John Wiley & Sons, Ltd.

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INTRODUCTION

Venom of social Hymenoptera is a complex and multifunctional secretion that covers a wide range of defensive necessities of colonies, sometimes containing thousands of individuals. This secretion is dispensed by means of a sting apparatus derived from the transformation of abdominal sclerites; it is produced by tubular (and convoluted) glands and stored in a reservoir.¹ The secretion is a product of a long evolutionary process from solitary species that use it as a fluid to paralyze preys for the rearing of their larvae, to social species that use it mainly for defensive purposes. Social wasps in particular, use the venom as an alarm and sexual pheromone,² as an antimicrobial agent³ and as a chemical weapon against invertebrate and vertebrate enemies.^{4,5} The venom is constituted of a mixture of several substances, which can be roughly divided into three main fractions: a low molecular weight (MW) volatile fraction, a medium MW fraction and a proteic fraction.⁶

From the medical point of view, the venom of stinging Hymenoptera has a noteworthy importance, as it can induce strong allergic reactions, up to anaphylactic shock and death, in sensitive patients.⁷ The allergic reaction is mainly caused by antigenic proteins, which in the social Vespidae are represented by three principal enzymes (phospholipase A, protease, and hyaluronidase) and a nonenzymatic protein^{6,8}

called *Antigen 5*; nevertheless, diffusion of these large molecules into the tissues of the victims is certainly facilitated by a set of smaller molecules, including peptides having MW not exceeding 3000 Da. Preventive treatment of allergic patients is made with a desensitizing immunotherapy based on the administration of increasing quantities of venom [Venom Immunotherapy, (VIT)]. A recent review on the argument, after observing that the estimated incidence of 40 fatal reactions each year in the United States of America is probably an underestimate, concludes that the efficacy of VIT (reported to be 95%) is greater than that of any other type of allergen immunotherapy.⁷ Notwithstanding the fact that the venom of various species presents cross-reactivity, it seems obvious that VIT could have more efficacy if carried out with the actual venoms, resulting in a positive skin test or radioallergosorbent test (RAST) result. This leads to the necessity to fully understand the chemical composition of the venom of the most common stinging Hymenoptera species. However, owing to the remarkable complexity and the species-specificity of this secretion, information is limited at present mainly to the antigenic proteins and the main pharmacologically active substances of the most common species.⁶ As a contribution to this research, we employed the matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique to determine the reference spectra (profiling) of the venom of some common Vespids (family Vespidae). Peptide mapping and spectra profiling using MALDI-TOF MS analysis and other MS techniques have already been made for characterizing the venoms of

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various organisms including arachnids, insects, jellyfish, snakes etc.^{9,10} In some cases, this approach has been used for establishing the phylogenetic relationships between various species belonging to the same taxonomical group (see, e.g. Wermelinger *et al.* for snakes of the genus *Bothrops* and *Crotalus*¹¹) or to highlight differences between individuals belonging to the same species (as Jabukowski *et al.* did for venomous fish-hunting *Conus* snails¹²). Regarding social Vespidae, MS techniques have been employed for the study and the characterization of the most important allergens of the venom of various species, although this was performed mainly on the basis of differences in their amino acid sequences (for a recent review see Kolarich *et al.*¹³).

The aims of our research were: (1) testing the suitability of the MALDI-TOF technique for the comparison of the venoms belonging to different species of social Vespids on the basis of their spectral profiles, (2) the collection of data that could be used for the study of the phylogeny of this group of insects and (3) the design of a quick and reliable analytical method for the quality control of venom preparations to be used in VIT. The MALDI technique requires very low amounts of substance and this allowed us to analyze, in a very short time, the venom of single individuals belonging to each examined species – this was important for treating the data with statistical tests controlling for intraspecies variations. In particular, we used ClinProTools 2.0 (CPT), a software until now used mainly in medical researches, primarily developed for proteomic analyses and for the individuation of disease markers in body fluids.^{14,15} In this paper, we report the main preliminary results obtained from analyses of the venom fraction ranging from 900 to 3000 Da collected from three European and one North American paper wasps (genus *Polistes*) and from the European hornet *Vespa crabro*. These species can be considered to be within the most common Vespids in the Mediterranean regions and the United States. We chose four species belonging to the same genus (*Polistes*) and one to a relatively phylogenetically distant one (*Vespa*), as we expected different degrees of differentiation in the chemical profile of their venoms. The analysis of the volatile fraction of the venom for some *Polistes* species has been reported by Bruschini *et al.*,^{16,17} whereas the comparative analysis of the higher MW proteic fraction is in progress.

EXPERIMENTAL

Species analyses

Polistes dominulus, *P. gallicus*, and *P. nimphus* are common paper wasps in the Mediterranean regions and belong to the subgenus *Polistes* s.s. The first one was introduced in the United States of America almost 20 years ago and here it became the dominant paper wasp species in the Eastern and Central States.¹⁸ *P. exclamans* is a North American species belonging to the subgenus *Aphanilopterus*.^{19,20} *V. crabro* is a hornet widespread in Europe, Asia, and North America, where it was introduced in the second half of the 19th century. For a review on social wasps biology, refer to Ross and Matthews.²¹

Wasps were captured from nests or in flight in the surroundings of Florence (Central Italy) and in the countryside around Houston (Texas, U.S.A.).

Chemicals

Methanol and acetonitrile were of chromatography grade and purchased from Riedel de Haen (Sigma Aldrich, Milan, Italy). Purified and deionized water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA.). Formic acid and trifluoroacetic acid (TFA) were purchased from Fluka (Sigma Aldrich, Milan, Italy). Centricon filter unit's vials with nominal MW cutoff of 3000 Da were purchased from Millipore (Bedford, MA, USA.). α -Cyano-4-hydroxycinnamic acid (HCCA) was obtained from Sigma (Sigma Aldrich, Milan, Italy). The matrix for MALDI-TOF experiments was a mixture of a saturated solution of HCCA in acetonitrile and 0.1% TFA in water (1 : 2, v : v).

Venom collection and sample preparation

Wasps were killed at low temperature (-20°C) and stored in a freezer. After dissection, the venom sacs were gently squeezed until the venom came out from the tip of the sting and was immediately collected in a thin glass capillary.

- Preliminarily, we performed several analyses on the venom from single specimens of *P. dominulus* (around 0.3 μl for each specimen) directly deposited on an AnchorChipTM 400 target (Bruker) followed by matrix application (0.35 μl of matrix solution HCCA 5 g/l in 1 : 1 acetonitrile : water containing TFA 0.1%).
- The venom obtained from single specimens of *P. dominulus* ($N = 20$) was diluted in 50 μl of 1 : 1 methanol : water containing TFA 0.1% and then centrifuged for 2 min at 4500 g. This dilution ratio was established after several dilution trials. We applied 1 μl of the supernatant on the MALDI plate, left it to dry and then applied 0.35 μl of matrix on the sample.
- In order to enhance the peak signal intensity in the mass range considered, the venom obtained from single specimens of *P. dominulus* ($N = 10$), after dilution (as above), was filtered using a Centricon unit (with a filter cutting above 3 kDa) and then 1 μl of the filtrate was applied on the AnchorChipTM target as described above. This same procedure was used for preparation of venom samples obtained from single individuals of the other two European species, *P. gallicus* ($N = 10$), *P. nimphus* ($N = 10$) and of the North American *P. exclamans* ($N = 7$) and of the European hornet *V. crabro* ($N = 9$).

The AnchorChipTM target, prepared with all the sample sets (as described in A, B and C), was analyzed using an automatic procedure available on the MALDI mass spectrometer in order to standardize the results.

Mass spectrometry analyses

Spectra were acquired using a MALDI Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion reflector mode; the accelerating voltage and the Ion Source 2 were set to 25.0 and 21.9 kV, respectively, and the delay time was 20 ns; 300 shots were automatically accumulated for each spectrum.

External calibration was performed using the Bruker Standard Peptide Calibration kit.

We performed also an internal calibration using the Flex AnalysisTM software by Bruker Daltonics, Bremen, Germany,

as we had the exact mass of two peptides (1854 and 1909 Da) that we recently identified in the venom of *P. dominulus*.³

Data processing

The procedure used for data processing with CPT was suggested by the software manual and is similar to that used by Zhang *et al.*¹⁵ for profiling potential biomarkers in the plasma of asthma patients. We first calibrated all the spectra (refer to the previous paragraph) and imported them to the CPT. The program calculates the areas of the most important peaks that account for statistical differences between the venom of the various species. These peaks are highlighted by the software, which uses them to generate a model to classify the spectra and eventually to assign any other new spectrum to a particular group. The settings we used for the analysis are reported in the tables. The values of the same peaks' areas were also used to perform a Stepwise Discriminant Analysis and to obtain graphs of the groups' distributions.

RESULTS AND DISCUSSION

The rapid analysis of the venom, consisting in its direct deposition on the MALDI plate followed by matrix application (see 'Venom collection and sample preparation A'), did not lead to any significant result. The samples appeared as a translucent surface, probably due to some interfering compounds (fats, sugars) present in the venom. These results are in agreement with previous findings (Dotimas and Hider²² on honeybee), where it was shown that at least 7% of venom dry weight is composed of sugars and phospholipids. Consequently, the samples for MALDI-TOF analysis were obtained by dilution of the venom samples (see 'Venom collection and sample preparation B'). Under these experimental conditions only the soluble compounds present in the supernatant were analyzed.

Furthermore, the supernatant solution was filtered through a 3 kDa cutoff Centricon unit and the filtrate (see

'Venom collection and sample preparation C'), enriched in medium MW components, was prepared for MALDI-TOF.

In order to obtain statistically significant profiles, MALDI spectra of two different sample sets (20 venom samples from *P. dominulus* prepared as in 'Venom collection and sample preparation B', and 10 venom samples from *P. dominulus* prepared as in 'Venom collection and sample preparation C') were processed by the CPT software. CPT generates an average spectrum of the two sample sets: the comparison of the data obtained by the two approaches described above (simple dilution approach *vs* Centricon-based approach) is reported in Fig. 1.

As it can be observed, the spectra are almost superimposable: the same ions in the range m/z 900–3000 are present, with some differences in relative abundance. In particular, by using the Centricon-based method, a higher abundance of the ions at higher m/z values is observed.

Setting a signal-to-noise threshold of 3.0 (on average spectrum) and a relative threshold base peak of 1.0%, the software found 33 peaks suitable for the following elaboration of models (Table 1); the model produced with the Quick Classifier procedure discriminated correctly 97.5% of the samples with 97.6% for cross validation (a procedure which minimizes the risk of overfitting producing a model that leaves out a fraction of the total number of samples) (Table 2). The two different sample preparations can therefore be easily distinguished by the analysis. For these reasons all further investigations, aiming at finding possible differences at a specific level, have been performed using the Centricon-based approach.

CPT was then used to compare the spectra obtained from the individual samples ($N = 46$) of the five different species considered. The software found 28 peaks suitable for the elaboration of discriminative models. Figure 2 reports the average MALDI spectra for the five different species, whereas Table 3 (I model) lists the main peaks for each species. The main similarities were found between European

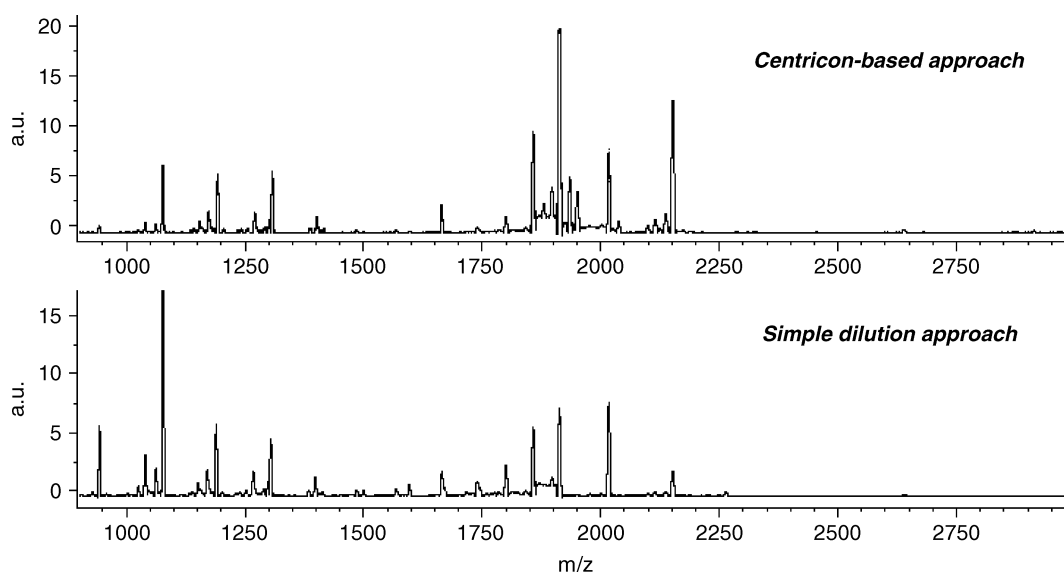


Figure 1. Average MALDI spectra of *P. dominulus* venom samples obtained from the venom, with the simple dilution approach ($N = 20$) and with the Centricon-based approach ($N = 10$).

Table 1. List of the 33 main MALDI-TOF peaks, ranging from 900 to 3000 m/z , identified by the CPT in two sets of specimens of *P. dominulus* venom (simple dilution approach vs Centricon-based approach) for the creation of the discrimination model. As $z = 1$, values of m/z are in equivalent Daltons

Peaks m/z		
927.39	1484.09	1886.42
941.52	1498.79	1895.63
1023.34	1567.26	1903.05
1038.03	1594.86	1911.25
1060.47	1663.2	1933.2
1075.6	1737.98	1949.24
1168.69	1798.66	2015.31
1187.62	1856.14	2113.19
1266.04	1870.28	2135.24
1296.72	1878.35	2150.27
1396.74	1737.98	2638.35

Table 2. CPT model for recognition of *Polistes dominulus* individuals' venom prepared with two different methods (simple dilution method and Centricon-based method) and for recognition of *P. dominulus*, *P. gallicus*, *P. nimphus*, *P. exclamans* and *V. crabro* individuals' venom (prepared according to the Centricon-based method) for MALDI-TOF analysis. CPT parameters used for model generation for method discrimination and species discrimination analyses were: Peak width: 0.01; Smoothing (width: 5 Da, cycles: 1); Average Peak List Calculation (Relative Threshold Base Peak: 0.01, Signal-to-Noise Threshold: 3, Limit Peak Number: false); Area Calculation (Integration Type: end point level); Peak Selection (Use All Peaks: true, Sort Mode: P -value tta); Model Generation (Algorithm: Quick Classifier); Cross Validation (Percent Leave Out: 20%, Number of Iterations: 10)

	Recognition capability (%)	Cross validation (%)
Method discrimination		
Overall	97.5	97.6
<i>P. dominulus</i> – simple dilution method ($N = 20$)	100	100
<i>P. dominulus</i> – Centricon-based method ($N = 10$)	95	95.24
Species discrimination		
Overall	96	94.43
<i>P. dominulus</i> ($N = 10$)	100	90.48
<i>P. gallicus</i> ($N = 10$)	90	90
<i>P. nimphus</i> ($N = 10$)	90	91.67
<i>P. exclamans</i> ($N = 7$)	100	100
<i>V. crabro</i> ($N = 9$)	100	100

Polistes, while *P. exclamans* and *V. crabro* show completely different spectral patterns.

The model produced by the software (using the Quick Classifier algorithm) discriminated correctly 98% (99.17% with cross validation) of the samples into the right species

Table 3. Main peaks ranging from 900 to 3000 Da found for the five different wasp species ($P.n = Polistes nimphus$, $P.d = P. dominulus$, $P.g = P. gallicus$, $P.e = P. exclamans$, $V.c = Vespa crabro$). Some of these peaks are used for comparison and discrimination of specimens belonging to the five species (I model) and some for the discrimination of the specimens belonging to the European *Polistes* species (II model)

Species Peak m/z	I model						II model		
	$P. n$	$P. d$	$P. g$	$P. e$	$V. c$		$P. n$	$P. d$	$P. g$
940	X	X	X						
977					X				
1075	X	X	X						
1117				X					
1239				X					
1266			X						
1298					X				
1301		X							
1331				X					
1362				X					
1376	X						X		
1395				X				X	
1471				X					
1496				X					
1507				X					
1584				X					
1776				X					
1784									X
1855	X	X							
1870			X						X
1896			X						
1910	X	X							
2048	X						X		
2089				X					
2149		X						X	
2185				X					
2224				X					

(Table 2). Using a more traditional multivariate statistical analysis (Stepwise Discriminant Analysis) applied to the 28 peaks identified with CPT, we obtained 100% of the samples correctly assigned to the right species with 99.9% of the variance in the data set explained by the two first discriminant functions (Function 1: Wilks' Lambda = 0.000, $\chi^2 = 786.04$, $P < 0.001$; Function 2: Wilks' Lambda = 0.000, $\chi^2 = 430.39$, $P < 0.001$). The chemical distances of the various species are highlighted by the graph of Fig. 3.

However, when we tried to classify the venom 20 specimens of *P. dominulus* treated with the simple dilution approach protocol ('Venom collection and sample preparation B') using the CPT discriminatory model described above, we obtained only ten of them correctly classified in the right species, while the other ten were erroneously assigned to the other two European *Polistes*.

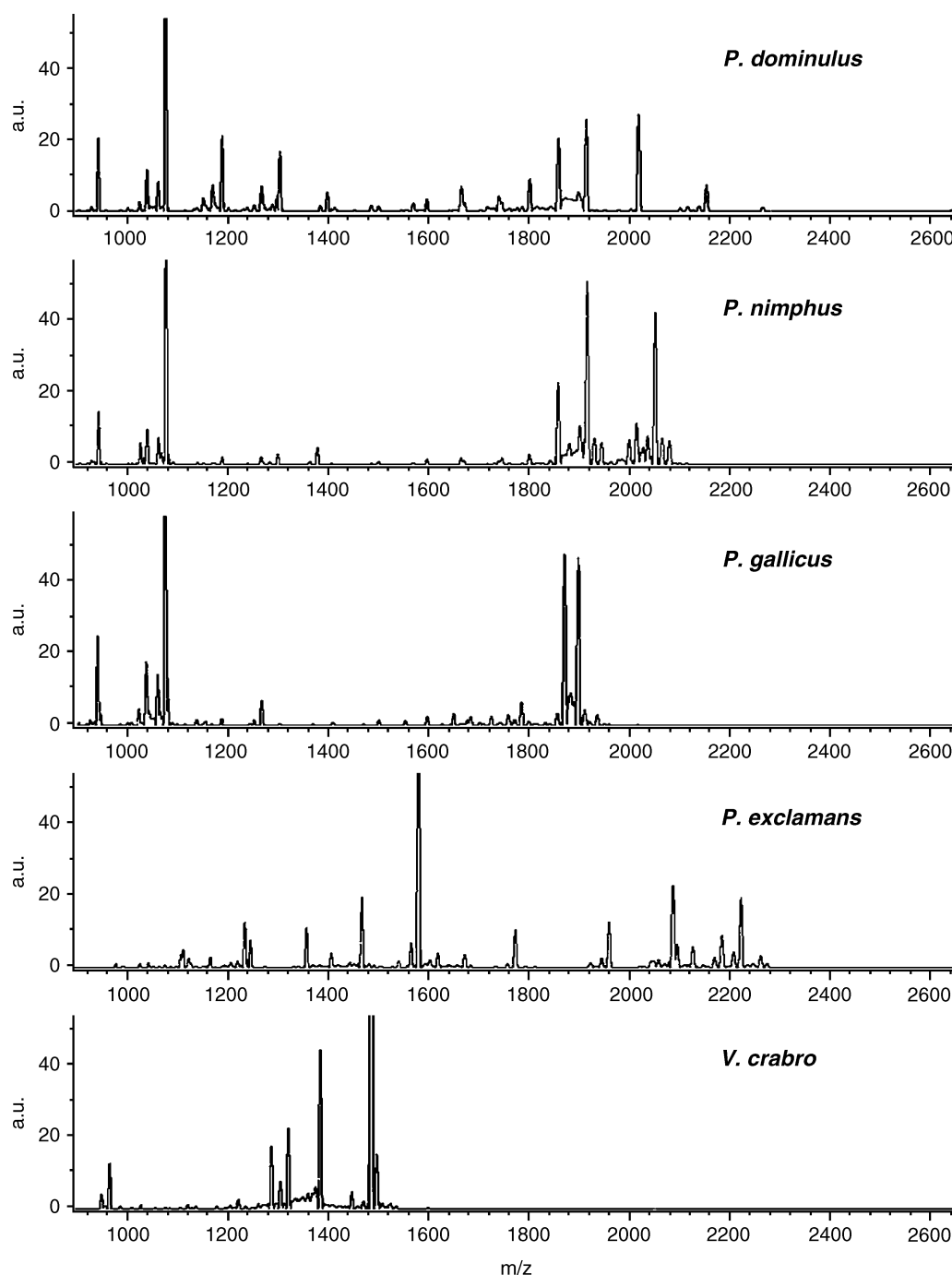


Figure 2. Average MALDI spectra of venom samples from the five examined species *P. dominulus*, *P. nimphus*, *P. gallicus* (10 samples each), *P. exclamans* (7 samples) and *V. crabro* (9 samples), obtained with the Centricon-based approach.

We then tried to obtain a CPT model only for the European *Polistes* species ($N = 30$). This time, however, we used the Gel View Facility supplied by the program in order to choose the most suitable peaks (i.e. the most representative peaks for each species) listed in Table 3 (II model) for a correct discrimination of the venom patterns. This is a rather subjective approach, but it is quite immediate and quick owing to the evident differences in the graphical representation (Fig. 4).

We used an alternative algorithm supplied by the CPT software (Genetic Algorithm), as it allows the use of the chosen peaks ('forcing' them into the model) to develop a

discriminatory model. This new approach allowed a complete discrimination among the European *Polistes* species (100% recognition capability both with and without cross validation) and classified correctly all the *P. dominulus* samples obtained with the simple dilution approach (20 out of 20).

Chemical analyses for the identification of the peaks contributing to the discrimination model are still in progress: for two of these substances we already determined their peptidic nature and their sequence, 1854 Da and 1909 Da in *P. dominulus*.³ The peptidic nature of some peaks of the other species was also confirmed, with the same technique as used by Turillazzi *et al.* (2006).³

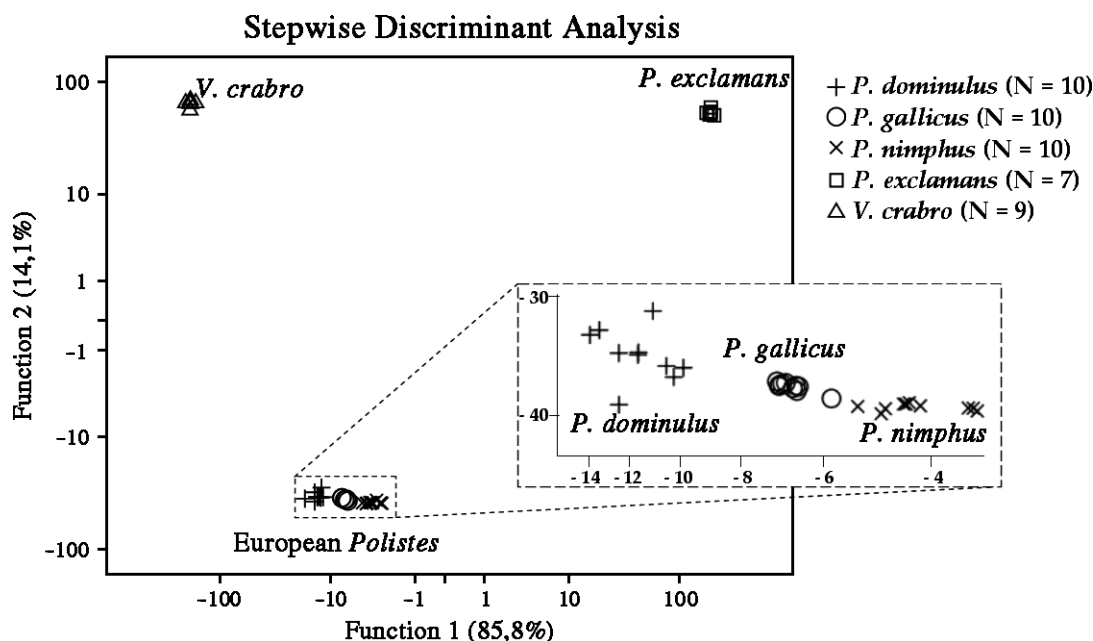


Figure 3. Plot of the scores of the two first Discriminant Functions obtained from the Stepwise Discriminant Analysis of the areas of 28 MALDI spectral peaks of the medium MW fraction of the venom of 46 wasps belonging to the five species examined.

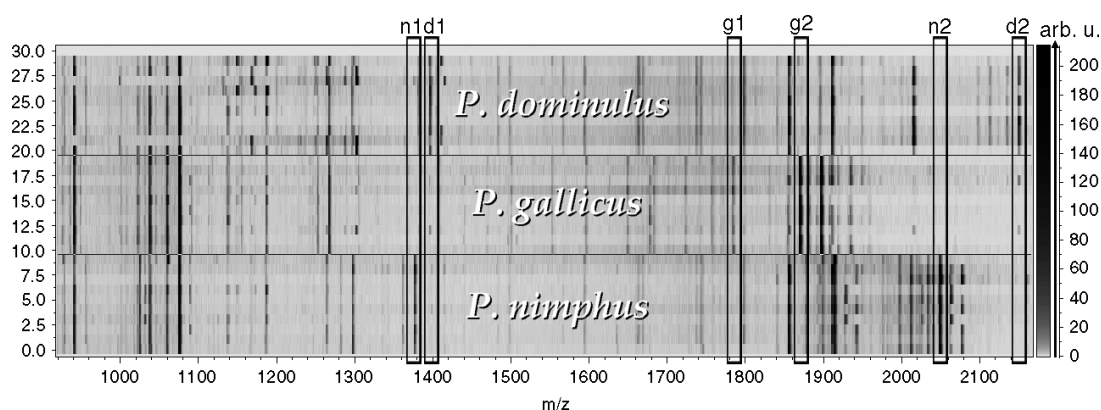


Figure 4. Gel View Facility supplied by the ClinProTools software in order to choose the most suitable peaks for discrimination of European *Polistes* venom. All the 30 samples of the three species analyzed are represented in the figure: the spectral pattern of each individual is shown as a horizontal line with color intensity standing for the relative abundance of the peaks. n1, n2, g1, g2, d1, d2 are the chosen representative peaks for *P. nimphus*, *P. gallicus* and *P. dominulus*, respectively, whose masses are reported in Table 3.

CONCLUSIONS

This research demonstrates that different species of social wasps have different venom composition in the MW fraction ranging from 900 to 3000 Da. Average spectral profiles of this fraction can be diagnostic for the identification of the species and the results are consistent with the relative taxonomical distances of the species. These differences are both qualitative and quantitative, and each species can be distinguished by a set of fundamental peaks. The identification of these peaks in future studies will be important for fully characterizing the chemistry of the venom of each species.

Differential preparations of venom can influence the corresponding spectral patterns but do not seriously affect the average profile and the classification of the species. This could be important also for quality control of commercial venoms before their use for VIT, assuming that the chemical

preparation maintains the medium MW fraction of the secretion.

The variations that we observed between venoms of individuals belonging to the same species deserve a deeper investigation, as they could be related to differences in age, caste, etc. This opens interesting questions about the possibility to document the chemical dynamics of the venom and its correlation with colonial organization of these social insects. MALDI-TOF technique coupled with the CPT software has proved to be a powerful analytical system for the determination of markers discriminating different groups of samples to be used in a wide range of studies.

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