1. Title page

Setae from the pine processionary moth (*Thaumetopoea pityocampa*) contain several relevant allergens

Running head: setae allergens

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Authors contributions:

Rodríguez-Mahillo AI, González-Muñoz M and Moneo I coordinated the work of all the co-authors, performed the SDS-PAGE, immunoblots, RP-HPLC, cloning and expression of the recombinant protein. They wrote the manuscript and contributed to the revision of the different versions of it.

Vega JM and García Ortiz JC interviewed the patients, performed the skin prick tests, collected the human sera and did the statistical job. They collected *T. pityocampa* larvae and extracted the larvae setae. They contributed to the revision of the different versions of the manuscript.

López JA and Camafeita E performed the MALDI MS-MS and *de novo* sequencing of the RP-HPLC fractions of the setae extracts. They contributed to the revision of the different versions of the manuscript.

Yart A and Roques A collected *T. pityocampa* larvae. They contributed to the revision of the different versions of the manuscript.

Kerdelhué C and Vogel H elaborated the *T. pityocampa* EST library and performed the comparison of the peptide sequences obtained by MALDI MS-MS and de novo sequencing with the library. They contributed to the revision of the different versions of the manuscript.

Toffolo EP, Zovi D and Battisti A collected *T. pityocampa* larvae and extracted the larval setae. They contributed to the revision of the different versions of the manuscript.

List of Abbreviations

EST: expressed sequence tag
MALDI: matrix-assisted laser desorption/ionization
MS: mass spectrometry
TOF: time of flight
RP-HPLC: Reversed Phase-High performance liquid chromatography

2. Abstract

Background: Pine processionary larvae produce urticating hairs (setae) that serve for protection against predators. Setae induce cutaneous reactions in animals and humans. The presence of toxic or allergic mechanisms is a matter of debate.

Objective: to detect the presence and characterize allergens in setae.

Patients, Material, Methods: Setae extracts were characterized by gel staining and immunoblot using sera from patients with immediate reactions and positive skin tests, as well as a rabbit antiserum raised against setae. Setae proteins were fractionated by HPLC. The most relevant allergen was analysed by MALDI-MS and its sequence was deduced from an EST bank.

Results: Setae contained at least seven different allergens. The most intense detection corresponded to a protein of around 14 kDa similar to thaumetopoein, a previously described protein with mast-cell degranulating properties. MALDI-MS based *de novo* sequencing provided a partial amino acid sequence different from that of the previously described allergen Tha p 1 and it was denominated Tha p 2. This allergen was detected by 61% of patients and it is therefore a new major caterpillar allergen.

Conclusions: Penetration of the setae from the pine processionary caterpillar delivers their allergenic content in addition to a mechanical or toxic injury.

5. Key words: *Thaumetopoea pityocampa*, allergy, allergen, caterpillar, setae, immunoblot, MALDI-MS, HPLC

Introduction

Lepidoptera are among the most common insects with around 175,000 described species worldwide (1). Accidental contact with the hairs/bristles of some larvae and adult moths induce symptoms that range from mild cutaneous lesions to severe systemic reactions, depending on the species involved, the severity of the contact, and the physical conditions of the victim (2). Larvae of the genus *Thaumetopoea* (Lepidoptera Nodontidae), commonly known as processionary moths, are found in Europe and close relatives are also found in Australia, Asia and Africa (1). *Thaumetopoea pityocampa* (pine processionary moth, PPM) and *Thaumetopoea processionea* (oak processionary moth) seem to be most relevant sensitizers in Europe (3-9). To protect themselves against natural enemies, processionary larvae evolved an urticating apparatus. The tegument of larvae at 4th (L4) and 5th (L5) larval instars exhibits different kinds of hair-like material; i.e., non-removable 'true' hairs and removable setae disposed on cuticular plates (called mirrors) on the dorsal surfaces of the first eight abdominal segments (10). The urticating symptoms are generally shown after contact with mature larvae, either alive or dead, and with airborne material produced by them (3, 10, 11). Usual reactions to processionary larvae appear on the contact areas, but systemic manifestations such as anaphylactic shock has been described as well (12-14). These kind of severe reactions suggest that an IgE-mediated mechanism can be operative, at least in some subjects. However, the presence of immune or toxic mechanism is still a question of debate (15-21).

The sensitising capacity of insects has been reported (22, 23). Allergic reactions after contact with biting or stinging insects are easy to understand, as the injection of a small amount of allergens can induce specific immune responses. However, the way in which processionary larvae can trigger immune responses remains unknown. Specific IgE has been measured using a crude extract of L5 larvae and the agreement between symptoms and

positive skin prick tests and/or in vitro determinations suggested that some of the allergens present in a crude larval extract are responsible for the allergic manifestations (15-21).

The aim of this work was to study if larval setae contain allergens and the different response patterns of the sera from subjects with clinical symptoms and positive skin prick tests.

The present work proves that the setae of the pine processionary larva contain different allergens that can be released on contact with the larva.

Patients, Material and Methods

Patients and skin prick tests

Eigthteen patients from a highly processionnary-infested area near Valladolid (Spain) showing immediate reactions to pine processionary larvae and a positive skin prick test were included. Skin prick tests were performed with a whole body and a setae extract, both from L5 (Bial-Aristegui Laboratory, Bilbao, Spain). The extracts were tested on 10 non-exposed subjects (5 atopic and 5 non atopic) as controls. Histamine dihydrochloride (10 mg/ml) and sterile 0.9% saline were used respectively as positive and negative controls. The response was considered positive if the mean wheal area was >7 mm^2 (wheal diameter >3 mm) compared with the negative control and measured 15 minutes after puncture. Prick response area was compared between larval and setae extract for each patient using a non-parametric Wilcoxon sign rank test. This study was approved by the Ethics Committee of the Hospital Universitario Río Hortega (Valladolid, Spain) and all patients included were asked to sign an informed consent.

Setae Collection

Colonies of *Thaumetopoea pityocampa* were collected in February 2009 from trees of *Pinus nigra* in Tregnago (Verona, Italy) when all the larvae were inside the nest, and immediately frozen at – 20 °C. The nests were then open in a hood and the L5, still frozen, transferred into vials in groups of 10. The vials were kept at – 20 °C until used for setae extraction. To prepare a sample of setae, a vial was taken inside a hood and the setae of each larva were manually removed with forceps under a stereomicroscope with a 40x magnification. Attention was paid to collect only the setae from the urticating apparatus, avoiding any contamination with true hairs and integument wounding. Best results detaching the setae were obtained with partially thawing larvae.

Protein extraction from the setae and crude larval extract preparation

The setae (0.6 g) were mixed with 0.6 ml of PBS and sonicated on ice for 10 seconds at 18 watts. The resulting mixture was centrifuged 10 minutes at 16,000 g. The supernatant was kept at -20 ºC until used. A crude larval extract was obtained as previously described (21).

Antiserum production

An anti-setae antiserum was obtained immunising rabbits with a whole setae extract sent to Biomedal SL (Seville, Spain). This immunogen was obtained washing twice 1,2 g of setae in PBS (2 ml) and sonicating the pellet with 2 ml of PBS. The slurry was allowed to settle for 5 minutes in order to discard large debris. Finally, the supernatant obtained was aspirated with a 1 ml pipette with a standard tip and the aspirated slurry was transferred to a new tube. Rabbits were immunized four times during a 2 month period.

SDS-PAGE and immunoblotting

Electrophoresis was carried out on 16% Tris-tricine minigels according to standard procedures. After electrophoresis, proteins were transferred to nitrocellulose by overnight diffusion, and the membranes were revealed using the anti-setae rabbit antiserum or the sera from the 18 patients shown in Table I and the corresponding antisera (anti-rabbit IgG, anti-human IgE or anti-human IgG) according to previously published methods (24, 25). Two non atopic patients' sera were used as negative controls.

High performance liquid chromatography (HPLC)

The setae extract (0.5 ml) was separated by reversed phase (RP)-HPLC on a Europa Protein 300 C4 column (20 x 0.46 cm; particle size 5 µm; Teknokroma, Barcelona, Spain).

Sample injection and elution was performed with an Agilent 1200 Series liquid chromatograph (Agilent Technologies, Inc., CA, USA). Elution was carried out with a linear gradient of ACN in 0.1% (vol/vol) TFA (0% to 5% for 10 minutes and 5% to 100% for 60 minutes, at a flow rate of 1 ml/min). Each fraction was tested for IgE-binding by immunoblot using a pool of positive sera.

Protein digestion and mass spectrometry

Fractions 44, 45 and 48 from the RP-HPLC separation were digested with modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 300 ng/µl at 37 ºC for 4 h. The digestion solutions were mixed with an appropriate matrix solution and the mixture was deposited onto a prestructured Matrix-Assisted Laser Desorption/Ionization (MALDI) probe (Bruker Daltonik, Fremont, CA, USA) (26) and allowed to dry at room temperature. Samples were analyzed in an Ultraflex MALDI-Time-Of-Flight (TOF)/TOF mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI-Mass Spectrometry (MS) and MALDI-MS/MS spectra as described previously (27).

Database searching and *de novo* sequencing

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search the National Center for Biotechnology Information (NCBI) non-redundant protein sequences database using the Mascot software (Matrix Science, London, UK) (28). Manual *de novo* sequencing was performed based on MALDI-MS/MS spectra from selected peptides (29). The so-obtained peptide sequences were submitted to the Basic Local Alignment Search Tool (BLAST) search algorithm at the NCBI.

Peptide N-terminal sulfonation and *de novo* sequencing

Derivatization with 4-sulphophenyl isothiocyanate (SPITC) (Sigma Chemical, St. Louis, MO, USA) of peptides from the digestion solutions of fractions 44, 45 and 48 from the RP-HPLC separation, was performed according to the method described by Wang et al. (30). Briefly, the above-described digestion solutions were incubated with 10 mg/ml SPITC in 20 mM sodium bicarbonate (Sigma Chemical) for 1 h at 55 ºC. The reaction was terminated by adding a small volume of 5% TFA. The resulting solution was vacuum-dried and resuspended in 20 µl of 0.1% TFA for cleanup with home-made reversed-phase columns (31) prior to MALDI analysis. Manual *de novo* sequencing was performed based on MALDI-MS/MS spectra from selected derivatized peptides (29).

Tha p 2 cloning

Peptide sequences obtained by MALDI-MS *de novo* sequencing were compared to the PPM Expressed Sequence Tag (EST) library stored at Centre de Biologie et Gestion de Populations (INRA Montpellier, France).

PCR primers TP2 DF (5'-GTCCCGCAACTAAGTGAGAAAGC-3') and TP2 DR (5'-TTACTAGGCCGAACAAGGACC-3') were designed upon the sequence of the mature protein (without the signal peptide) obtained from the translated EST sequence that matched the Tha p 2 peptides. PCR conditions were 94ºC for 1 min, 10 cycles of 94ºC for 30 sec, 70ºC, decreasing 1ºC/cycle, for 30 sec and 72ºC for 30 sec, 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec and a final elongation step of 72ºC for 5 min. PCR products were cloned in the vector pGEM T easy (Promega) and sequenced.

Results

Characterisation of the patients' immune response

Table 1 shows the clinical data and Fig 1 the IgE recognition pattern of 18 patients whose serum samples were used. There was no statistical difference in weal size between the two extracts (Wilcoxon test; P= 0.11). No positive skin prick tests were found in the control group.

A high proportion of the positive skin prick test sera recognized allergens in the crude extract (Fig 1). The most frequently detected allergen in the crude larval extract was a 15 kDa protein that could correspond to the major allergen Tha p 1 previously described by our group (21). Patients also detected at least seven different allergens on the setae extract with an allergen of around 14 kDa being the most frequently recognised (11/18). The subjects that were positive in the crude larval extract were also positive in setae, with the exception of patient 9. This serum had a strong detection of several high molecular weight allergens in the crude extract, but none in the setae extract. This patient had a strong positive skin prick test when the crude larval extract was tested, but gave negative results with the setae extract (Table 1).

Detection of specific IgG to proteins present both in the crude larvae and setae extracts was negative with exception of serum #3, which was strong positive to an antigen with a molecular weight similar to Tha p1 (Fig 2). This patient showed a long history of severe reactions after exposure to larvae, some of them requiring treatment in emergency units.

Fractioning by HPLC

A reversed-phase HPLC separated at least 70 fractions from the setae extract used. After lyophilisation, all the fractions were resuspended in 100 µl of PBS and studied by immunoblot using the rabbit antiserum and human sera as a source of IgE antibodies. Figure

3A shows the protein profile of the fractions with the highest A_{280}. The rabbit antiserum detected the presence of antigenic proteins mainly in three different peaks (fractions 44, 45 and 48) having the same molecular weight (Fig 3B). Two other proteins with a slightly higher molecular weight were detected with lower intensity. The use of the sera from sensitized patients (Fig 3 C) revealed that all the proteins detected by the rabbit anti-setae antiserum were in fact allergens. In addition, two other proteins of lower molecular weight bound specific IgE from the serum pool and must be considered as allergens present in setae.

MALDI-MS based *de novo* sequencing

Given that the Mascot searches performed with combined MALDI-MS and MALDI-MS/MS data obtained from the tryptic digests of fractions 44, 45 and 48 (Fig 3) failed to assign any statistically significant peptide or protein matches, a manual *de novo* sequencing approach based on MALDI-MS/MS data was performed. This produced the putative sequences NNLFNLGSVAGDILSR at mass-to-charge ratio (m/z) 1689.892, KAEEAIDLTYQEK at m/z = 1537.79, SYSQSYSYVQCTQDSECNGCWK at m/z = 2737.08 and DGCHVSFGCHK at m/z = 1303.53.

Since these sequences showed no significant similarity to Tha p 1 or any other protein by BLAST, the digested fractions were derivatized with SPITC , which permitted to confirm the m/z 1689.892 sequence assignment. This suggested that the above sequences originated from a new caterpillar allergen which was registered as Tha p 2 (UniProt Knowledgebase accession number: P86360).

Tha p 2 cloning and expression

Peptide sequences obtained by proteomic approaches were compared to the PPM EST sequences library. They matched to mRNAs codified by contigs 28088I454 and 28087I454.

The translation of these contigs (Fig 4A) resulted in a theoretical protein of 115 amino acids with a predicted signal peptide of 15 amino acids deduced using SignalP 3.0 (www.cbs.dtu.dk/services/SignalP). The predicted mature Tha p 2 is a 11.024 kDa protein of 100 amino acids. The alignment of the peptides sequenced and the predicted Tha p 2 is shown in Fig 4B.

Discussion

The nature of the urticating substances and their mechanism of action in the etiology of the reactions to larval Lepidoptera have been a matter of controversy**.** Substances with enzymatic properties have been described (32). Pine and oak processionary larvae have an urticating apparatus known as mirror, which may release up to one million of setae to the air in order to protect the larvae from their predators (33). The seta is very sharp in the distal part and carries backward spikes in the proximal part in order to facilitate the penetration of the setae in the skin or mucosa of the attacking animal. Lamy et al (34) extracted soluble proteins from setae and isolated a 28 kDa urticating protein composed of two subunits of 13 and 15 kDa known as thaumetopoein. This protein is present in large quantity in the glands that produce the setae (35, 36), it is recognized by a rabbit antiserum produced against setae (as in our case) and it has mast cell degranulating properties suggesting a nonspecific mechanism of inflammation (36). However, the same group describe several years later that foresters heavily exposed to pine caterpillars can be sensitized to proteins extracted form setae, and that three of the four positive cases had specific IgE against thaumetopoein (37). They describe two allergens, one of them having a molecular weight of 18 kDa and the other migrated as a band with molecular weight higher than 45 kDa. According to our present data, thaumetopoein and our main setae allergen could correspond to the same protein, but unfortunately no information about amino acid composition of thaumetopoein is available. In initial experiments performed by us, crude larval extracts did not activate basophils from non-sensitized controls (data not shown), a fact that argued against a non-specific mechanism as described for thaumetopoein, but the non-specific activation of thaumetopoein was tested on peritoneal mast cells instead of basophils (36).

The present study demonstrated that setae contain a complex mixture of at least 70 proteins, among them seven allergens that are delivered to humans by intradermal injection.

Previous experimental work in rats has demonstrated that intradermal administration of allergen allows production of specific IgE after booster injections 1000 times lower than by the oral route (38). Larval allergens are delivered to the skin by penetration of the setae, which have minute amounts of protein contained in a chitinaceous structure, and this way of administration seems to be the second relevant fact that could polarize the immune system to a T helper 2 (Th2) response. Chitin is a recognition element for tissue infiltration by innate cells implicated in allergic and helminth immunity (39). The role of chitin in allergic reactions due to its ability to produce tissue infiltration with IL-4 and IL-13-expressing cells, including Th2 cells, eosinophils and basophils after chitin exposure has been recently described (38) and it has been suggested that exposure to chitin might be the primary external determinant in allergy development (40). In this sense, Fagrell et al (33) recently reported that, following local application of setae of the northern pine processionary moth *Thaumetopoea pinivora*, setae penetrated in the outer skin layer and remained in the skin for up to 3 weeks. During all this time period, the setae could release allergens that could enhance an immune response on the affected individual. It is noteworthy that specific IgG was not found in the majority of our patients and suggested that processionary caterpillars induce a predominant IgE-mediated immune response in humans.

In conclusion, PPM setae have to be considered as a major source of allergens. As they can be delivered by various types of contact with the larvae as well as dispersed by wind over considerable distances (41), the risk for humans and animals is high in areas infested by these insects, such as forests, parks and gardens. In infested rural areas of the Mediterranean region, up to 12% of people may show reactions to PPM (42). However, PPM may be of even more concern in the near future. Global warming is largely favouring the survival of this winter-developing insect in more northerly areas where it was unable to develop before. Thus,

PPM is expanding towards urban, highly-populated areas such as the Paris Basin where more and more colonies are establishing via natural dispersal as well as through accidental translocations by humans (43). Therefore, the allergen content of the setae constitutes an emerging serious health hazard.

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Figure legend

Figure 1: IgE-immunoblot of the 18 positive sera from patients shown in Table 1 studied in the same order as presented in the table, using a crude larval extract (A) and the setae extract (B). Two unrelated human sera were used as a control (#19 and #20).

Figure 2: IgG-immunoblot of the 18 positive sera from patients shown in Table 1 studied in the same order as presented in the table, using a crude larval extract (A) and the setae extract (B). Two unrelated human sera were used as a control (#19 and #20).

Figure 3: HPLC of the setae extract. Coomassie stained SDS-PAGE (A), rabbit polyclonal (B) and human IgE (C) immunoblots of selected fractions of the setae extract after reversed-phase HPLC separation.

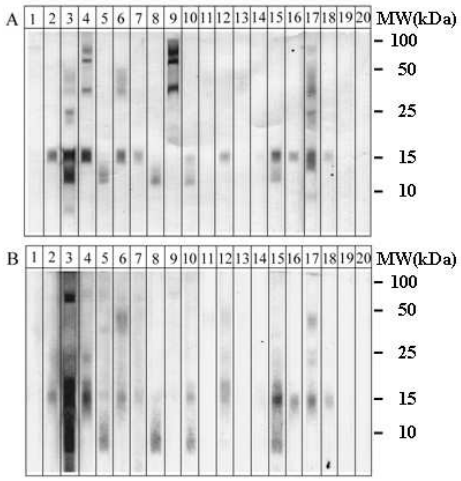
Figure 4: A: Sequences of contigs 28088I454 and 28087I454 and their translation to protein; **B:** Alignment of the theoretical sequence of Tha p 2 and the peptides obtained by MALDI-MS *de novo* sequencing of the 14 kDa allergen isolated from the setae extract by RP-HPLC. Tha p 2= sequence stored at *T. pytiocampa* EST library. 1, 2, 3, 4= sequences obtained by MALDI-MS *de novo* sequencing

Table I. Clinical data of the patients

Patient	Sex	Age	Atopy status	Symptoms	Cutaneous lesions distribution	Crude larval Prick area (mm^2)	Setae Prick area (mm^2)
1	m	27	Atopic dermatitis	AU, CU, E	Neck,face, extremities	50	28
2	m	43	R-C-A (pollens, ephitelia)	AU	Neck,face, extremities	13	50
3	m	14	-	AX (AU, CPD, E, D)	Neck,face, extremities	20	NT
4	f	20	R-C-A (pollens, ephitelia), food allergy	AX (AU E, C, R, D)	Neck, extremities, face, trunk	20	28.3
5	f	32	-	AU, E	Neck, extremities, face, trunk	154	38.5
6	m	16	-	AU, E	Neck, extremities, face, trunk	113	133
7	m	20	R-C (pollens, fungi, mites)	AU	Extremities	13	28
8	m	38	R-C (pollens, ephitelia), food allergy	AU, APD, E	Neck, face extremties	13	13
9	m	27	R-C-A (ephitelia, pollens, mites), food allergy	AU	Extremities, trunk	177	7
10	f	27	-	AU, CU, E	Neck	28	113
11	f	65	R-C (pollens)	AU	Neck	20	29
12	f	64	-	AU, E	Neck, extremities, face, trunk	20	50
13	f	21	R-C (pollens, ephitelia)	AU, E	Neck, face, extremities,	20	50
14	m	44	R-C (pollens, mites)	AU, E	Neck, face, extremities	13	20
15	m	34	R-C (pollens)	AU	Neck, extremities	28	64
16	m	37	RC (pollens, mites)	AU, C	Neck, trunk, extremities	7	20
17	m	73	R-C-A (pollens, mites), food allergy	AU, CU, E, C	Neck, face, extremities	64	20
18	m	44	-	AU, CU	Neck, trunk, extremities	0	38.5

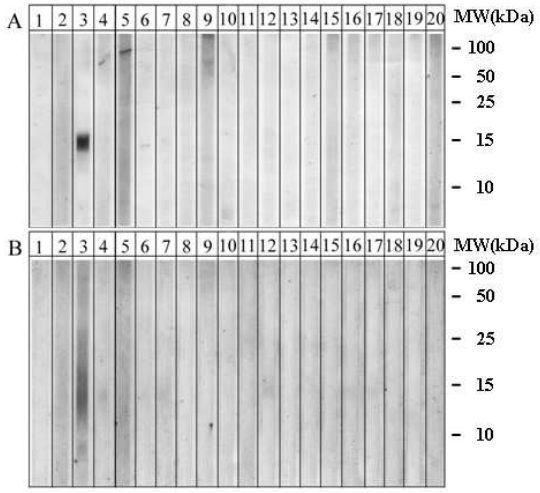
M= male, F= female, AU= airborne urticaria, APD= airborne papular dermatitis, A= asthma, CU= contact urticaria, CPD= contact papular dermatitis, E= edema, AX= anaphylaxis, C=conjunctivitis, R= rhinitis, D= dyspnoea; NT= non tested

Figure 1



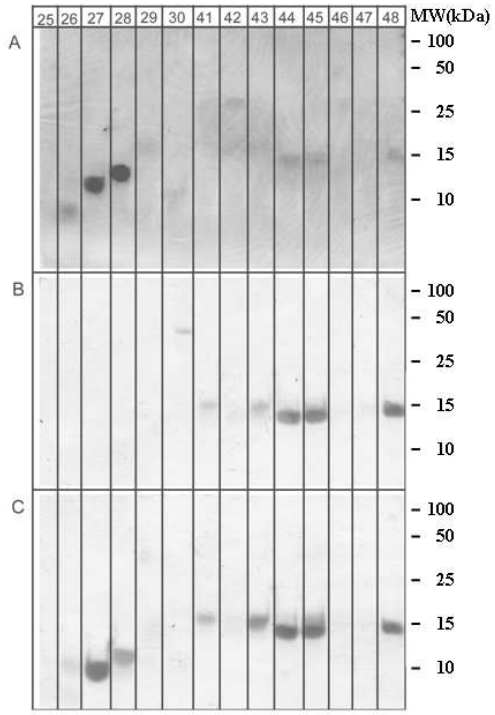
190x275mm (96 x 96 DPI)

Figure 2



190x275mm (96 x 96 DPI)

Figure 3



190x275mm (96 x 96 DPI)

Figure 4

A

```
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                                                  M  K  L  L  I
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 F  A  T  L  I  A  L  S  S  S  V  P  Q  L  S  E  K  A  E  E
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 D  I  L  S  R  D  G  C  H  V  S  F  G  C  H  K  G  Y  C  W
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 K  S  Y  S  Q  S  Y  S  Y  V  Q  C  T  Q  D  S  E  C  N  G
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 C  W  K  C  G  G  P  C  S  A  -  -
acatgattaaatggtgtatttaagagttcgtact
```

B:

```
Tha p 2         MKLLIFATLIALSSSVPQLSEKAEEAIDLTYQEKNNLFDLGSVAGDILSRDGCHVSFGCH 60
1               ------------------------------------------------DGCHVSFGCH 10
                                                                 **********
3               ------------------KAEEAIDLTYQEK---------------------------- 13
                                   *************
4               ---------------------------NNLFNLGSVAGDILSR---------- 16
                                            ****:***********

Tha p 2         KGYCWAGCGNPTNPWSWGENWCYTTKSYSQSYSYVQCTQDSECNGCWKCGGPCSA 115
1               K---------------------------------------------------- 11
                *
2               ------------------------SYSQSYSYVQCTQDSECNGCWK------- 22
                                         **********************
```

204x210mm (72 x 72 DPI)