

Identification of olive pollen allergens using a fluorescence-based 2D multiplex method

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Abstract

Olive (*Olea europaea* L.) pollen is a major health concern in the Mediterranean countries and some olive growing regions in America and Australia. The molecular variability of pollen allergens constitutes a handicap for commercial extract standardization, which is the base of current diagnosis and vaccination procedures. In this paper, we report a time- and plant material-saving multiplex detection method for the rapid and simultaneous analysis of Ole e 1, Ole e 2 and Ole e 5 allergen polymorphism on a single blot. This method combines high-resolution 2DE techniques with high-sensitive fluorescence-based detection methods. Using this strategy, we were capable to identify a higher number of allergen forms compared with classical 1D approach. The use of fluorescent probes and the increased resolution of 2D blots avoided overlapping effects, and allow estimating the amount of individual allergen forms. In addition, the pattern and identity of the IgE-reactive proteins of either a population or individual patients allergic to olive pollen was also effortlessly determined in a single additional step. This flexible method might be extended to a higher number of olive allergens and cultivars, and is also applicable to other allergogenic plant species and sources.

Introduction

Olive (*Olea europaea* L.) pollen is a main cause of seasonal respiratory allergy (i.e. pollinosis) in those regions of the world where this agronomic species is intensively cultivated [1]. The allergenicity of olive pollen relies on a number of allergenic proteins, twelve of which (Ole e 1 to Ole e 12) have been identified and characterized to date [2]. Most olive pollen allergens are polymorphic and exhibit several IgE-reactive forms on both 1D and 2D immunoblots [3-5]. This molecular polymorphism is mostly caused by point substitutions in the amino acid sequence. Thus, up to 59 and 39 different amino acid positions in the Ole e 1 and Ole e 2 sequences, respectively, showed intra-, inter-cultivar variations, or both [4, 6-

8]. Post-translational modifications (e.g. glycosylation) [4] and multimeric forms [3] also contribute to this molecular variability. Both the composition of the glycan and the presence of microheterogeneities affecting the epitope structure of Ole e 1 might influence its allergenic potency [9]. In addition to this qualitative diversity, some olive pollen allergens show quantitative differences depending on the genetic origin (i.e. cultivar) of pollen [10]. The expression levels of olive pollen allergens might be also affected by climatic parameters such as temperature and rainfall [11].

Pollen allergy diagnosis and specific immunotherapy (SIT) are based on the use of protein extracts prepared from commercially available pollen. The standardization of these preparations is essential to warrant efficiency and safety in the diagnosis and SIT procedures. The quantitative and qualitative variability found in natural allergenic sources such as pollen is a bottleneck for extract standardization and might lead to mistakes in allergy diagnosis and limited success in vaccination treatments in polysensitized patients [10, 12]. The standardization methods usually determine the overall potency of the IgE response to pollen protein extracts in a given population. Since pollen contains a mixture of major and minor allergens, there is a risk that minor allergens may be underrepresented in the extract [13]. In this scenario, the allergenic response will be dominated by major allergens, limiting the reaction to minor allergens, since not all patients are allergic to them. Therefore, it is important to quantify and evaluate the allergenic activity of each particular allergen form present in commercial extracts that are used for clinical purposes.

Methods based on electrophoretic separation of proteins and immunoblotting are particularly helpful for standardization [14]. Thus, specific antibodies allow detecting and quantifying allergen forms in non-standardized protein extracts. The resulting data can be used, in turn, to design adequate dosage schemes and for a better interpretation of the dose-therapeutic response of major and minor allergens. However, colorimetric multiplex methods

are time-consuming and present a number of disadvantages including the production of blot replicas (which compromises normalization and makes comparison between bands difficult) [15], and additional stripping and reprobing steps (which result in poor labeling) [16-17].

In a previous work, we developed a novel multiplex 1D Western blotting method for the simultaneous detection and quantification of four distinct olive pollen allergens [18]. This method introduced two major methodological novelties. First, unconjugated Fab (Fragment antigen-binding) fragments were used for blocking primary antibodies (Abs) raised in the same host species. Secondly, allergenic epitopes were simultaneously detected using commercial fluorochrome-conjugated secondary antibodies. In the present paper, we have developed a high-resolution multiplex system by coupling 2D electrophoresis and immunoblotting techniques with fluorescence-based detection procedures. The method has been validated for quantification of the different forms of three olive pollen allergens (Ole e 1, Ole e 2 and Ole e 5), as well as for IgE-reactive protein profiling purposes. These allergens were chosen on the basis of their differential allergenic relevance, their molecular features and the availability of antibodies (Supporting Information Table 1).

2 Material and methods

2.1 Plant material

Mature pollen grains of olive (*Olea europaea* L. cvs. 'Picual', 'Arbequina' and 'Lucio') were harvested from dehiscent anthers by vigorous shaking of flowering shoots inside large paper bags. Sampling was carried out from discrete trees of the germplasm collection of the CIFA “Venta del Llano” in Mengíbar (Jaén, Spain). Pollen samples were sieved through an appropriate set of meshes to remove floral debris and processed fresh or stored at -80°C until use.

2.2 Antibody and allergen production

The mouse anti-Ole e 1 mAb (10H1) was kindly provided by Dr. Carlos Lahoz (Fundación Jiménez Díaz, Madrid, Spain). The anti-Ole e 2 polyclonal antibody (PoAb) was produced by immunization of a rabbit with a keyhole limpet hemocyanin (KLH)-linked synthetic peptide ('N'- AQSATFPQFKPEEM) designed from the Ole e 2 amino acid sequence. The anti-Ole e 5 PoAb was produced by immunization of a chicken as described above, using a synthetic peptide ('N'- VGEDGTAAINIVDKC) designed from the Ole e 5 amino acid sequence. The specificity of all antibodies was experimentally confirmed in previous works [19-21].

The rOle e 1 allergen was kindly provided by Dr. Mayte Villalba (Universidad Complutense Madrid, Spain) [6]. A recombinant human profilin 1 (rPFN1, UniProt accession no. P07737) was purchased from Abcam (catalogue no. ab87760; Cambridge, UK), and a Cu-Zn superoxide dismutase (SOD) purified from horseradish was purchased from Sigma-Aldrich (catalogue no. S4636; St. Louis, MI, USA).

2.3 Olive pollen allergic patients' sera

Serum samples from three olive pollen allergic patients were obtained at the Allergy Service of University Hospital San Cecilio, in Granada (Spain). The selected patients had a clinical history of rhinitis and/or asthma and were immunoCAP- (Phadia, Uppsala, Sweden) and skin prick test (SPT)-positive to olive pollen protein extracts. Serum samples were individually frozen in liquid nitrogen and stored at -80°C until use.

2.4 Preparation of crude pollen protein extracts

Crude pollen protein extracts were prepared from olive pollen as previously described [4]. Total protein content was estimated using the 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.5 Two-dimensional multiplex Western blotting

Pollen samples containing 75 µg of total protein were applied to 10 cm polyacrylamide IPG strips (pH 3–10NL, Bio-Rad, Hercules, CA, USA) by in-gel rehydration at 30 V for 12 h. Focusing was conducted at 20 °C in an Ethan IPGphor 3 Cell (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as follows: 300 and 1,000 V for 1 h each followed by a linear increase from 1,000 to 10,000 V, and finally 10,000 V to give a total of 40 kVh. Reduction and alkylation steps were performed as previously described [22]. Protein separation in the second dimension was carried out in 4-20% Mini-PROTEAN® TGX precast gels (Bio-Rad, catalogue no. 456-1093) at 180 V for 1 h at 4°C using a Mini-PROTEAN Tetra Cell (Bio-Rad). Protein markers (Fermentas, Vilnius, Lithuania, catalogue no. SM0441; Bio-Rad, catalogue no. 161-0373) were co-electrophoresed in the second dimension.

Simultaneous detection of rOle e 1, rPFN1 and SOD proteins was carried out by blotting proteins onto a PVDF membrane in a Trans-Blot Turbo Transfer System (Bio-Rad) using the Trans-Blot Turbo Transfer Pack (Bio-Rad, catalogue no. 170-4157) according to the manufacturer's instructions. The resulting membrane was first blocked for 2 h with a TBS solution containing 0.3% (v/v) Tween 20 and 10% (w/v) skimmed dried milk. Then, the membrane was simultaneously incubated with the three primary Abs diluted in blocking solution (Table 1) for 2 h at room temperature, following by incubation for 1 h with the corresponding secondary antibody (Table 1) diluted in TBS buffer containing 0.3% Tween 20. Secondary antibodies were distinctly labeled with the DyLight 488, 549 or 633 fluorochromes (Agrisera AB, Vännäs, Sweden). Finally, the membrane was rinsed in the same buffer four times for 5 min each. Experiments were carried out in triplicate. Negative controls included the preimmune sera corresponding to the immunization with Ole e 1, Ole e 2 and Ole e 5 allergens.

2.6 Imaging and quantitation analysis

After washing, 2D blots were left to dry at room temperature in the dark and scanned with a Pharos FX Plus Molecular Imager (Bio-Rad) using the Quantity One v4.6.2 software (Bio-Rad). Settings of image acquisition were maintained across all the experiments. Blots were sequentially scanned at a resolution of 50 μm with the 488 (blue), 532 (green), and 635 nm (red) lasers, which correspond to the excitation wavelengths of DyLight 488, 549 and 633 fluorochromes, respectively. The emitted fluorescence was imaged using the appropriate emission filters. For presentation purposes, we assigned a false color to each channel.

A calibration curve was built for each allergen using a dilution series (1, 2, 4 and 8 μg) of the purified protein. Thus, samples containing 1, 2, 4 and 8 μg of rOle e 1, rPFN1 and SOD proteins were diluted in Laemmli sample buffer [23], denatured by heating at 100 $^{\circ}\text{C}$ for 15 min and cooled on ice. Samples were then loaded on 12% polyacrylamide vertical precast gels (Bio-Rad, catalogue no. 456-1043) and SDS-PAGE was conducted at 180V for 1 h using a Mini-PROTEAN Tetra Cell (Bio-Rad). Gels were electrophoresed in duplicate, the first gel being stained with CBB according standard procedures. The second gel was used for immunodetection purposes, using antibody dilutions as above (Table 1).

The intensity of each protein band in the resulting 1D blot was quantified using the “% adjusted volume” tool of the Quantity One 4.6.2 software (Bio-Rad). This parameter is defined as the sum of the intensities of the pixels inside the volume boundary $\times$ area of a single pixel (in mm^2) minus the background volume. Blots were carried out in triplicate ($N=3$) and band intensity means $\pm$ SD were computed and plotted against the protein amount (in μg) using the Excel software (Microsoft, USA).

2.7 Analysis of IgE reactivity

The IgE reactivity was assayed on the 2D blots produced above using a pool of three olive allergic patients' sera and imaged by chemiluminiscence using an alkaline phosphatase (AP)-conjugated goat anti-human IgE antibody (Sigma-Aldrich), as described previously [18]. Moreover, a single patient's serum was also independently tested using 'Arbequina' and 'Lucio' pollen protein extracts.

3 Results and discussion

3.1 The fluorescence-based 2D multiplex detection method allows simultaneous detection and differentiation of a high number of olive pollen allergen forms

We first developed a reliable and reproducible fluorescence-based multiplex method that allows simultaneous detection and differentiation of olive pollen Ole e 1, Ole e 2 and Ole 5 allergen forms on 2D blots (Fig. 1A-D). The distribution pattern of Ole e 1 forms obtained after labeling with 10H1 mAb in 'Picual' was similar to that previously reported [4-5]. Additionally, we could identify eight minor spots (Fig. 1A, spots 1a, 1b, 1c, 1f, 1q, 1r, 1u and 1v), likely because fluorescent labeling techniques are more sensitive than the enzyme-based colorimetric detection method used in those pioneer studies. Pollen grains collected from 'Arbequina' and 'Lucio' trees displayed 10 and 16 Ole e 1 forms on 2D protein maps, respectively (Fig. 1E-F). Three new Ole e 1 forms were described in these two cultivars, two of them (1k and 1p) being specific of 'Lucio' while form 1e was only detected in 'Arbequina'. Three Ole e 1 "spot trains" in a molecular weight range of 18-22 kDa were clearly distinguishable on 2D maps (Fig. 1A), which correspond to non-glycosylated, mono-glycosylated and hyper-glycosylated forms of the allergen, respectively [3-5]. Ole e 1 forms showed *pI* values in a pH range between 5.0 and 8.5. This shift in *pI* in either the acidic or basic direction is likely caused by the existence of both microheterogeneities in the amino acid sequence and variations in the composition of the N-glycan moiety [4, 7]. In quantitative

terms, the total content of this allergen was much higher in 'Picual'. Spots named as 1j, 1m and 1o were the most abundant Ole e 1 forms in 'Picual' and 'Lucio' pollen, whereas 1e, 1m and 1o were the most relevant forms in 'Arbequina'.

The number of forms detected for Ole e 2 and Ole e 5 allergens was significantly lower compared with Ole e 1. All the cultivars showed three reactive spots on 2D blots after incubation with anti-Ole e 2 PoAb, although they varied in their pIs (Fig. 1). All these spots corresponded to five different Ole e 2 forms, two of which (2a and 2e) were new forms of this allergen. Ole e 2a and 2e forms were identified on the basis of their distinct isoelectric point and migration pattern. Thus, it was possible to easily discriminate the cultivars analyzed since 2a, 2c and 2e forms were specific of 'Lucio', 'Picual' and 'Arbequina' pollen, respectively. These five Ole e 2 forms appeared clustered in two different protein bands on a multiplex 1D map [18, 24]. The anti-Ole e 5 PoAb bound to a train of two major and two minor immunoreactive spots in 'Picual' pollen. These four Ole e 5 forms were previously described as a single allergen on a multiplex 1D map [18, 24]. Both the number of forms and the total amount of Ole e 5 were lower in 'Arbequina', and the allergen was almost undetectable in 'Lucio'. A new form of this allergenic protein (5c) was also identified in 'Arbequina'. Control reactions lacked of reactivity in all cases (data not shown).

Overall, our experiments resulted in a higher number of olive pollen allergen forms identified on 2D blots compared with a previous multiplex 1D approach [18]. Thus, current data reveal the existence of at least 22 Ole e 1, and 5 Ole e 2 and Ole e 5 allergenic forms, respectively. In parallel to its higher resolution, this 2D multiplex system saves time and plant material because additional stripping and reprobing steps and replicas are not necessary [17].

The reproducibility of the enzyme/substrate-based detection methods is low and the alignment of different blots is often difficult. Multiplex methods overcome this latter problem as long as signals do not overlap on the blot. All these factors make protein quantification

challenging. By producing the specific standard, it is possible to estimate the concentration of any protein [25]. To explore the potential use of this strategy for allergen quantification from non-standardized extracts, we used rOle e 1, rPFN1 and nSOD proteins as Ole e 1, Ole e 2 and Ole e 5 standards, respectively. We first tested their purity and cross-reactivity by 1D SDS-PAGE and Western blot (data not shown). rOle e 1 and rPFN1 proteins were visible after Coomassie staining as a unique protein band of 21 and 14 kDa, respectively. rOle e 1 was recognized by 10H1 mAb, while the anti-Ole e 2 PoAb cross-reacted with rPFN1. A major protein band of about 16 kDa was recognized by the anti-Ole e 5 PoAb in Western blot experiments. An additional minor protein of 14.5 kDa that co-purified with nSOD was visible after Coomassie staining, but this protein did not cross-react with the anti-Ole e 5 Ab.

In a second step, we made dilution series of each protein standard, which were electrophoresed in 1D polyacrylamide gels (Fig. 2A) and blotted on PVDF membranes (Fig. 2B). The resulting fluorescent blots were scanned and the mean intensity of each band was computed and plotted against the protein amount loaded on gels. We found a positive logarithmic correlation between both parameters in all the three proteins analyzed (Fig. 2C). Thus, the fluorescence intensity increased in a linear manner when the amount of protein standard increased up to four micrograms and then gradually reached a plateau. Fluorescence crosstalk was minimized using probes with non-overlapping emission wavelengths.

In order to validate this method, we quantify the amount of each allergen form present on 'Picual' 2D blots by measuring the intensity of fluorescence (adjusted volume %) of each immunoreactive spot using the "volume contour" tool of the Quantity One software (Fig. 3A). The fluorescent signal overlapped in spot pairs 1i+1j, 1l+1m and 1n+1o, and hence the corresponding allergenic forms could not be individually quantified. Ole e 1 j, 1m and 1o forms showed the highest values of intensity, whereas Ole e 5e, Ole e 1a, 1b, 1c, 1f, 1q and 1r spots showed the lowest intensity levels (Fig. 3B). Further, we calculated the amount of

protein contained in each spot by extrapolating the fluorescence intensity data (Fig. 3C). We found a good positive correlation between both parameters (Spearman's $\rho = 0.777$, $P = 0.000$). Consequently, spots named as Ole e 1j, 1m and 1o were also the most abundant allergen forms in the 2D protein map.

According to our data, this 2D multiplex system allows estimating simultaneously the amount of different allergens since detection does not depend on enzyme/substrate kinetics and fluorescent signals can be spectrally separated on a single blot. Moreover, the high resolution capacity of the 2DE techniques allows a better separation of allergen forms on blots and hence individual quantitative estimates. However, quantification of allergens using this 2D multiplex method should not be considered as a substitute but a complementary method to the highly sensitive *in vitro* ELISA-derived standardization techniques [26]. Top-down Fourier Transform (Orbitrap) MS-based methods allow relative and absolute quantification of intact proteoforms with the greatest specificity and accuracy [27-28] and might be applied for allergen quantification as well. This approach is also more costly and time-consuming compared with our 2D multiplex method, and needs high-qualified operators and expensive equipment. Therefore, its general use for routine allergen standardization needs to be evaluated.

3.2 IgE-binding protein profiling of olive pollen allergic patients

Atopic patients often show differential reactivity to pollen protein extracts depending on the cultivar assayed [10, 29]. For a more efficient vaccination, allergic patients should be treated with those allergenic proteins producing high levels of IgE [30-31]. To assess whether this 2D multiplex method can be used to identify allergic patients' IgE-binding proteins [18], we designed two different experiments. Firstly, the blots resulting from multiplex detection of 'Picual' Ole e 1, Ole e 2 and Ole e 5 allergens were reprobed with a pool of three olive pollen

allergic patients' sera using a chemiluminiscence detection system. Patients' sera reacted with more than 80 protein spots with molecular weights ranging from 14 to 50 kDa, which covered most of the pH gradient (Fig. 4A). We could easily confirm the identity of some of the IgE-binding proteins by overlapping both multiplex and chemiluminescence images obtained from the same blot (Fig. 4B). Thus, the most prominent IgE-binding spots undoubtedly corresponded to the several forms of Ole e 1 allergen identified in the multiplex experiment. Four other spots matched well with Ole e 5 forms but only Ole e 2b reacted with the IgEs from the tested sera (Fig. 4B). Additionally, a number of spots with molecular weights in the range of 26-50 kDa did not match with any of the three allergens analyzed, so they likely correspond to other allergenic proteins from olive pollen.

Secondly, we investigated whether the IgE-binding protein pattern of a particular allergic patient is depending on the genetic origin (i.e cultivar) of the protein extract assayed. To accomplish this objective, we randomly chosen a single serum and determined its IgE reactivity against 'Arbequina' (Fig. 4C-D) and 'Lucio' (Fig. 4E-F) pollen protein extracts. Yet again, it was fast and easy to match and identify the most prominent IgE-binding allergens. Based on these data, and independently of the olive cultivar considered, we might conclude that this patient is strongly allergic to Ole e 1 but does not show sensitization to Ole e 2 or Ole e 5 allergens. However, since patterns differed in the number, intensity and coordinates of spots on 2D maps, it is suggested that IgE reactivity to other olive pollen allergens might depend on the allergenic source (i.e. cultivar) to which the patient is exposed.

Finally, another putative clinical application of this 2D multiplex method would be the identification of natural allergen hypoallergenic forms (i.e. allergens with reduced allergenic activity and retained immunogenicity). The use of these hypoallergenic protein forms might improve immunotherapy in allergic patients [32]. For this purpose, it will be necessary to explore the vast olive germplam [33].

3.4 Concluding remarks

Here, we report a two-step fluorescence-based 2D multiplex detection method for the rapid and simultaneous analysis of the polymorphism of three olive pollen allergens, namely Ole e 1, Ole e 2 and Ole 5, on a single blot. This high-resolution multiplex system easily differentiates overlapping allergenic forms, which can be also individually estimated. In this way, it would be possible to determine the contribution of each allergen form to the total allergenic potency of a protein extract. The resulting data can help in turn to design adequate dosage schemes and to elucidate the dose-therapeutic response relation of major and minor allergens. Subsequently, the individual IgE-binding protein pattern of allergic patients can be also achieved reprobing the same blot in a single additional step. The identity of the patient's IgE-reactive proteins can be easily confirmed by matching spots from multiplex and IgE detection-derived images. This information can be useful to choose which allergens are better for immunotherapy in each individual. This multiplex method described here is versatile since it can be also used with other allergogenic plant species (e.g. grasses) or sources (e.g. foods). Future work will expand this method to a higher number of allergenic proteins in order to cover the entire olive pollen allergen spectrum, and to other olive varieties that are also widely and extensively cultivated.

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References

- [1] Liccardi, G., D'Amato, M., D'Amato, G., *Int. Arch. Allergy Immunol.* 1996, *111*, 210–207.
- [2] Villalba, M., Rodríguez, R., Batanero, E., *Methods* 2014, *66*, 44–54.
- [3] Villalba, M., Batanero, E., Lopez-Otín, C., Sánchez, L. M., Monsalve, R. I., González de la Peña, M. A., Lahoz, C., Rodríguez, R., *Eur. J. Biochem.* 1993, *216*, 863–869.
- [4] Castro, A. J., Bednarczyk, A., Schaeffer-Reiss, C., Rodríguez-García, M. I., Van Dorsselaer, A., Alché J. D., *Proteomics* 2010, *10*, 953–962.
- [5] Zienkiewicz, K., García-Quirós, E., Alché, J. D., Rodríguez-García, M. I., Castro, A. J., in: Jiménez-López, J. C. (Ed.), *Current insights in pollen allergens*, InTech Open, Rijeka, Croatia 2012, pp. 41–56.
- [6] Villalba, M., Batanero, E., Monsalve, R. I., González de la Peña, M. A., Lahoz, C., Rodríguez, R., *J. Biol. Chem.* 1994, *269*, 15217–15222.
- [7] Hamman-Khalifa, A. M., Castro, A. J., Jiménez-López, J. C., Rodríguez-García, M. I., Alché, J. D., *BMC Plant Biol.* 2008, *8*, 10.
- [8] Jiménez-López, J. C., Morales, M., Castro, A.J., Volkmann, D., Rodríguez-García, M. I., Alché, J. D., *PLoS ONE* 2012, *7*, e30878.
- [9] González, E., Villalba, M., Lombardero, M., Aalbers, M., Van Ree, R., Rodríguez, R., *Mol. Immunol.* 2002, *39*, 93–101.
- [10] Castro, A. J., Alché, J. D., Cuevas, J., Romero, P. J., Alché, V., Rodríguez-García, M. I., *Int. Arch. Allergy Immunol.* 2003, *131*, 164–173.
- [11] Morales, S., Castro, A. J., Rodríguez-García, M. I., Alché, J. D., in: Jiménez-López, J. C. (Ed.), *Current insights in pollen allergens*, InTech Open, Rijeka, Croatia 2012, pp. 57–70.
- [12] Canonica, G. W., Baena-Cagnani, C. E., Bousquet, J., Bousquet, P. J., Lockey, R. F., Malling, H.-J., Passalacqua, G., Potter, P., Valovirta, E., *Allergy* 2007, *62*, 317–324.

- [13] Van Ree, R., *J. Allergy Clin. Immunol.* 2007, *119*, 270–277.
- [14] Van Ree, R., *Allergy* 1997, *52*, 795–805.
- [15] Patton, W. F., *Electrophoresis* 2000, *21*, 1123–1144.
- [16] Kaufmann, S. H., Ewing, C. M., Shaper, J. H., *Anal. Biochem.* 1987, *161*, 89–95.
- [17] Krajewski, S., *Methods Mol. Biol.* 2009, *536*, 463–472.
- [18] Morales, S., Castro, A. J., Jiménez-López, J. C., Florido, F., Rodríguez-García, M. I., Alché J. D., *Electrophoresis* 2012, *33*, 1–8.
- [19] Lauzurica, P., Gurbindo, C., Maruri, N., Galocha, B. Díaz, R., González, J., García, R., Lahoz, C., *Mol. Immunol.* 1988, *25*, 329–335.
- [20] Morales, S., Jiménez-López, J. C., Castro, A. J., Rodríguez-García, M. I., Alché, J. D., *J. Microsc.-Oxford* 2008, *231*, 332–341.
- [21] Zafra, D., Jiménez-Quesada, M. J., Traverso, J.A., Corpas, F. J., Rodríguez-García, M. I., Alché J. D., *Microsc. Microanal.* 2012, *18*, 33–34.
- [22] Görg, A., Postel, W., Günther, S., *Electrophoresis* 1988, *9*, 531–546.
- [23] Laemmli, U. K., *Nature* 1970, *227*, 680–685.
- [24] Morales, S., Castro, A. J., Salmerón, C., Marco, F. M., Rodríguez-García, M. I., Alché, J. D., in: Jiménez-López, J. C. (Ed.), *Current insights in pollen allergens*, InTech Open, Rijeka, Croatia 2012, pp. 27–40.
- [25] Fradelizi, J., Friederich, E., Beckerle, M. C., Golsteyn, R.M., *Biotechniques* 1999, *26*, 484–486.
- [26] Arilla, M. C., Eraso, E., Ibarrola, I., Algorta, J., Martínez, A., Asturias, J. A., *Ann. Allergy Asthma Immunol.* 2002, *89*, 83–89.
- [27] Nikolov, M., Schmidt, C., Urlaub, H., *Methods Mol. Biol.* 2012, *893*, 85–100.
- [28] Kelleher, N. L., Thomas, P. M., Ntai, I., Compton, P. D., LeDuc, R. D., *Expert Rev. Proteomics* 2014, *11*, 649–651.

- [29] Geller-Berstein, C., Arad, G., Keynan, N., Lahoz, C., Cardaba, B., Waisel Y., *Allergy* 1996, 51, 356–359.
- [30] Valenta, R., Vrtala, S., Laffer, S., Spitzauer, S., Kraft, D., *Allergy* 1998, 53, 552–561.
- [31] Valenta, R., Lidholm, J., Niederberger, V., Hayek, B., Kraft, D., Grönlund, H., *Clin. Exp. Allergy* 1999, 29, 896–904.
- [32] Vrtala, S., *Allergy* 2008, 63, 299–309.
- [33] Ganino, T., Bartolini, G., Fabbri, A., *J. Hort. Sci. Biotech.* 2006, 81, 319–34.

Table 1. Antibodies and dilutions used for 2D Multiplex Western blotting and IgE-binding experiments.

Target	Primary antibody (source)	Dilution	Secondary antibody	Dilution
Ole e 1	Mouse anti-olive Ole e 1 mAb (Lauzurica <i>et al.</i> 1988)	1:5,000	DyLight 633-labeled goat anti-mouse IgG	1:10,000
Ole e 2 (Profilin)	Rabbit anti-olive Ole e 2 PoAb (Morales <i>et al.</i> 2008)	1:10,000	DyLight 549-labeled donkey anti-rabbit IgG	1:10,000
Ole e 5 (Cu/Zn SOD)	Chicken anti-olive Ole e 5 PoAb (Davids Biotechnologie)	1:100	DyLight 488-labeled goat anti-chicken IgG	1:10,000
Olive pollen allergens	Sera from olive pollen allergic patients	1:100	AP-conjugated goat anti- human IgE	1:10,000

Figure 1. (A-D) Fluorescence-based 2D multiplex detection of 'Picual' pollen Ole e 1, Ole e 2 and Ole e 5 allergen forms on a single blot. Fluorescent signals derived from Ole e 1 (blue color), Ole e 2 (red) and Ole e 5 (green) detection can be individually captured (A-C) or merged in a single image (D). (E-F) Multiplex detection of 'Arbequina' (E) and 'Lucio' (F) pollen Ole e 1, Ole e 2 and Ole e 5 allergen forms. Seventy five micrograms of total protein per sample were loaded. Protein markers are displayed on the left (Figs. 1A and 1E).

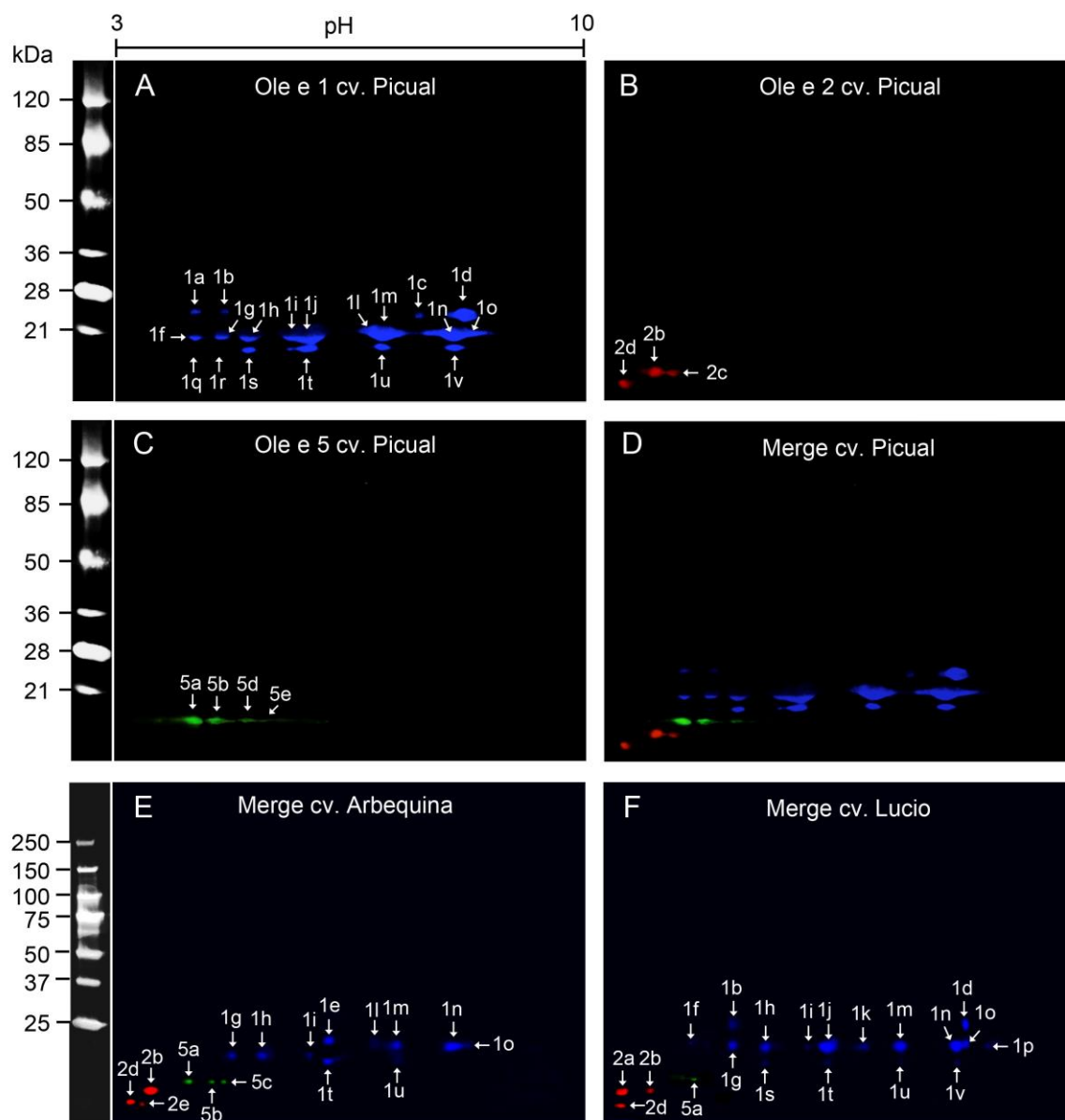


Figure 1

Figure 2. Calibration curves for rOle e 1, rPFN1 and SOD protein standards. (A) Coomassie-stained polyacrylamide gels of rOle e 1, rPFN1 and SOD protein dilution series. (B) Immunoblots as in A probed with the anti-Ole e 1 (left), anti-Ole e 2 (middle) and anti-Ole e 5 (right) antibody. (C) Calibration curves corresponding to rOle e 1, rPFN1 and SOD protein standards. The intensity of each band (% adjusted volume) is plotted against the protein amount (μg).

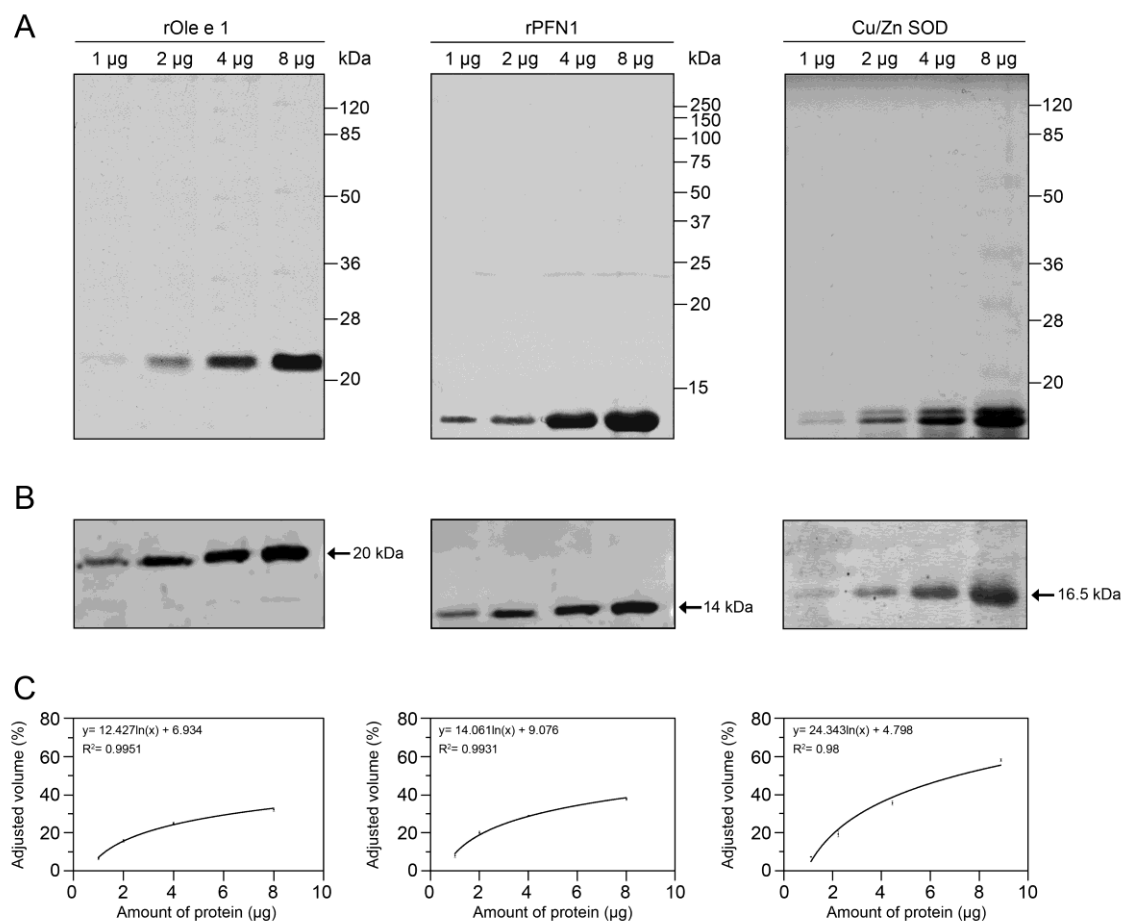


Figure 2

Figure 3. Quantification of 'Picual' pollen Ole e 1, Ole e 2 and Ole e 5 allergen forms detected on the 2D blots corresponding to three independent experiments. The fluorescent labeling intensity for each spot was measured in a densitometer and expressed as the average adjusted volume (% A), or the amount of protein (in μg , B).

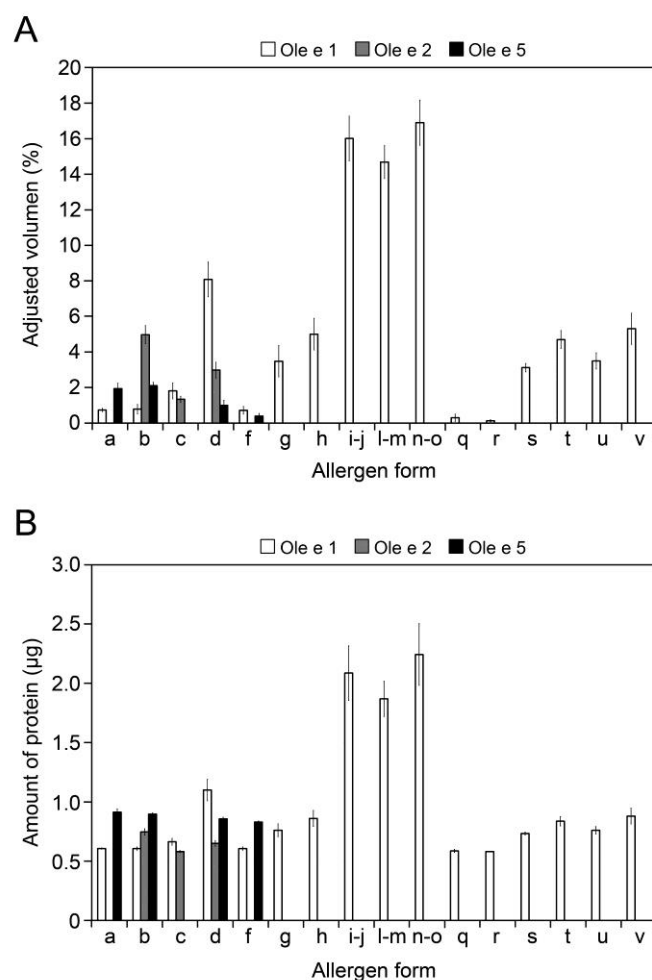


Figure 3

Figure 4. (A-B) IgE-binding analysis by 2D Western blotting of olive pollen total proteins from 'Picual' cultivar. The blot used for multiplex detection of Ole e 1, Ole e 2 and Ole e 5 allergens was reprobbed with a pool of sera from three patients allergic to olive pollen (A). Overlap of IgE-reactive and multiplex protein spots are shown in (B). (C-F) IgE-binding analysis by 2D Western blotting of 'Arbequina' (C-D) and 'Lucio' (E-F) pollen total proteins. The blots used for multiplex detection of Ole e 1, Ole e 2 and Ole e 5 allergens were reprobbed with a single patient's serum (C and E). Overlap of IgE-reactive and multiplex protein spots are shown in (D and F). Protein markers are displayed on the left (Figs. 4A, C and E).

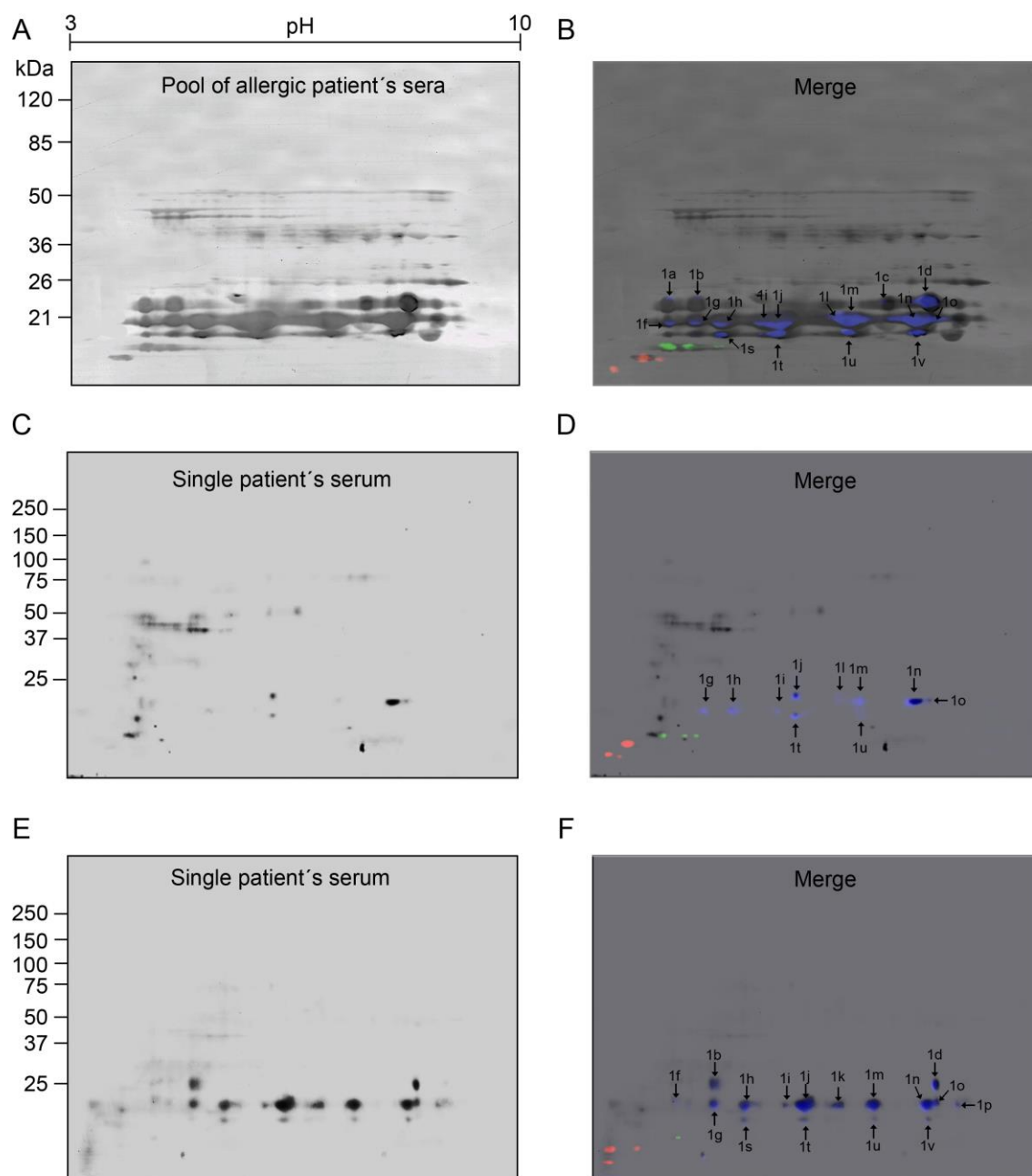


Figure 4

Supporting information

Table 1. Allergenic relevance and molecular features of the olive pollen allergens chosen for this study.

Allergen	Prevalence ¹ (%)	Size ² (kDa)	Molecular polymorphism	Cross-reactivity
Ole e 1	80	18-21	Very high	<i>Oleaceae</i>
Ole e 2	15	14-16	High	Panallergen
Ole e 5	39	16	Medium	NR

¹ Data from Villalba *et al.* (2014) Methods 66: 44–54; ²Molecular weights by SDS-PAGE; NR, not reported.