Biomolecular identification of allergenic pollen: a new perspective for aerobiological monitoring?

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Background: Accurate and updated information on airborne pollen in specific areas can help allergic patients. Current monitoring systems are based on a morphologic identification approach, a time-consuming method that may represent a limiting factor for sampling network enhancement.

Objective: To verify the feasibility of developing a real-time polymerase chain reaction (PCR) approach, an alternative to optical analysis, as a rapid, accurate, and automated tool for the detection and quantification of airborne allergenic pollen taxa.

Methods: The traditional cetyl trimethyl ammonium bromide–based method was modified for DNA isolation from pollen. Taxon-specific DNA sequences were identified via bioinformatics or literature searches and were PCR amplified from the matching allergenic taxa; based on the sequences of PCR products, complementary or degenerate TaqMan probes were developed. The accuracy of the quantitative real-time PCR assay was tested on 3 plant species.

Results: The setup of a modified DNA extraction protocol allowed us to achieve good-quality pollen DNA. Taxon-specific nuclear gene fragments were identified and sequenced. Designed primer pairs and probes identified selected pollen taxa, mostly at the required classification level. Pollen was properly identified even when collected on routine aerobiological tape. Preliminary quantification assays on pollen grains were successfully performed on test species and in mixes.

Conclusions: The real-time PCR approach revealed promising results in pollen identification and quantification, even when analyzing pollen mixes. Future perspectives could concern the development of multiplex real-time PCR for the simultaneous detection of different taxa in the same reaction tube and the application of high-throughput molecular methods.

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INTRODUCTION

Respiratory diseases, such as allergic rhinitis, conjunctivitis, and asthma, are distributed worldwide. A meta-analysis[1] estimated prevalences of 400 million people with allergic rhinitis (in 2006) and 300 million with asthma (in 2004) in a world population of 6.4 billion to 6.5 billion (Population Reference Bureau, World Population Data Sheet). Furthermore, Bauchau and Durham[2] estimated that approximately 45% of European adults do not have a diagnosis of allergic rhinitis. Pollen allergens are 1 of the major sources of respiratory disease. Diffusion of pollen allergens by ambient air is strictly related to the composition, spatial distribution, and density of allergenic taxa in an area and to meteorologic variables, such as wind stress, temperature, and humidity.[3]

Individual prevention measures are strongly recommended to control the symptoms of respiratory disease.[4] Some measures, however, can be adopted by patients only if accurate and updated information on the air pollen load is available. Reports and forecasts for the public (eg, http://www.aaaai.org/nab/ and http://www.polleninfo.org/) are produced by analyzing data from aerobiological monitoring centers, such as that in the Trentino area (northern Italy) since 1989. The applied standard for sampling and counting airborne pollen grains and fungal spores (UNI 11108:2004) identifies pollen grains by means of visual recognition of specific morphologic characteristics[5] and subsequent counting using an optical microscope. Correct application of the procedure is time-consuming and requires specialized personnel. The cost of these requirements is a limiting factor for sampling network improvement, which could give more precise information about the pollen load in specific areas. Nowadays, the possibility of easily isolating and studying genomic DNA can help biologists overcome the obstacles of traditional approaches for the identification and classification of plant taxa. Analysis of DNA sequence polymorphism, in particular, may be applied to different fields,[6] including land plant phylogenesis[7] and diagnostics.[8] With the advent of real-time polymerase chain reaction (PCR), detection and quantification of target DNA have been combined into a single reaction; therefore, various rapid, sensitive, and accurate assays can be elaborated.

The aim of this study is to verify the feasibility of developing a real-time PCR technique, an alternative to optical analysis, as a tool for the detection and quantification of airborne allergenic pollen taxa. This could lead to a rapid, accurate, and automated procedure that would allow an increase in sampling site distribution, useful to represent the variability especially in orographically and vegetationally complex regions.

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METHODS

Study Area

This study was performed in Trentino, a subalpine region of northern Italy extending from 45° 40′ to 46° 30′ north latitude and from 10° 30′ to 11° 50′ east longitude, with a surface area of 6.207 km^2; the elevation ranges from 65 to 3,764 m above sea level, with most (47%) of the area being 1,000 to 2,000 m above sea level. Consequently, the landscape is characterized by many phytoclimatic types, varying from sub-Mediterranean holly-oak woods to continental Swiss stone pine woods.[9]

Plant Species Selection

Plant taxa were selected on the basis of their allergic relevance and their presence in local flora (Table 1). Definition of the taxonomic level requested for the analysis was resolved by evaluating 2 main issues: (1) the target of allergic tests used to diagnose disease in individuals with allergy, depending on its turn in the pathologic response to allergens, which may be common in the same genus or family (eg, Poaceae[10]), and (2) morphologic characteristics of single pollens and the consequent identification level achievable under microscopic evaluation.[11]

Sample Collection, Preparation, and Storage

Pollen and leaf samples of each plant species were collected at 3 different sites with the aim of including natural genetic variation. Leaf tissues were sampled to obtain an easily available DNA template for development of the real-time PCR assay. Pollens were sampled after single-species flowering time, soon after anther dehiscence. They were desiccated and stored at a low temperature (4°C).[12] Samples of young leaves were collected from the same individual plants and were kept at −80°C.

Suspensions were prepared from stored pollen samples with different concentrations of 1 or 2 pollen taxa (50:50), selecting those that show an overlap in flowering time. The pollen content of suspensions was evaluated using a microscopic Fuchs-Rosenthal counting chamber. Simulated routine samples were prepared by spreading collected pollen onto an aerobiological tape (Melinex; DuPont Teijin Films Luxembourg SA, Luxembourg City, Luxembourg), coated with silicon-based adhesive (Lanzoni s.r.l., Bologna, Italy), aiming to reproduce samples collected using a Hirst-type volumetric device.

Table 1. Allergenic Pollen Species Studied and the Level of Classification Required From the Analysis

Family	Species	Required classification level
Betulaceae	*Alnus glutinosa*	Genus
	Alnus incana	Genus
	Betula pendula	Species
Compositae	*Artemisia vulgaris*	Family
Corylaceae	*Corylus avellana*	Species
	Ostrya carpinifolia	Species
Cupressaceae	*Cupressus arizonica*	Family
	Cupressus sempervirens	Family
	Thuja orientalis	Family
Oleaceae	*Fraxinus excelsior*	Genus
	Fraxinus ornus	Genus
	Olea europaea	Species
Poaceae	*Anthoxanthum odoratum*	Family
	Dactylis glomerata	Family
	Lolium perenne	Family
	Phleum pratense	Family
	Poa annua	Family
Urticaceae	*Parietaria officinalis*	Species

Setup of a DNA Extraction Protocol

DNA was extracted from leaf (0.1 g) and pollen (0.01–0.1 g) following the protocol of Doyle and Doyle[13] modified as described herein. Aerobiological tape spread with pollen was cut into small pieces of approximately 0.5 cm^2. Leaf tissues were ground using a manual mortar and liquid nitrogen and were stored at −20°C. Free and on-tape pollen grains and leaf tissues were incubated at 60°C for 45 minutes with cetyl trimethyl ammonium bromide buffer containing 0.3 mg/mL of proteinase K and 0.4% sodium dodecyl sulfate. Complete DNA extraction from immobilized pollen grains was evaluated by labeling the resumed tape with a DNA-specific probe (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich, Milan, Italy)[14] and by verifying the absence of fluorescent signals using a microscope. Isolated DNA was fluorometrically quantified using PicoGreen solution (Invitrogen, Carlsbad, California) and BioTek Synergy2 Multi-Detection Microplate Readers (BioTek Instruments Inc, Winooski, Vermont).

Identification and Sequencing of Suitable Taxon-Specific DNA Regions

A bioinformatics analysis was performed to identify taxon-specific DNA sequences. The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database was, therefore, queried for single- or low-copy nuclear genes or genomic sequences encoding non-repetitive elements. Performing a BLAST analysis[15] against the non-redundant nucleotide Viridiplanteae database, taxon specificity of selected DNA sequences was first evaluated in silico. Whenever this approach was not successful, a bibliographic search was performed.

Identified DNA regions were PCR amplified and sequenced by means of primers designed as described herein. Two to four nanograms of amplified DNA was used for every 100 base pair to be sequenced in both directions. The PCR products were purified using ExoSap-IT (Amersham Pharmacia Biotech, Uppsala, Sweden) and were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) in a GeneAmp PCR System 9700 (Applied Biosystems). After precipitation, the sequencing products were mixed with 10 μL of Hi-Di Formamide (Applied Biosystems) and were separated by means

of capillary electrophoresis in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The resulting data were analyzed using Sequencing Analysis version 3.7 (Applied Biosystems) and ChromasPro version 1.3 (Technelysium Pty Ltd, Tewantin, Australia). Alignment of amplicon sequences was performed using BioEdit version 5.0.6 (Hall, 1999).

PCR Primer and TaqMan Probe Design

Primers and TaqMan probes were designed using Primer Express version 2.0 (Applied Biosystems), were manually checked using Oligo Analyzer version 3.1 (IDT, http://eu.idtdna.com), and were synthesized by Sigma-Aldrich Diagnostic (St Louis, Missouri). The taxon specificity of each primer pair was evaluated by means of conventional PCR and gel electrophoresis using leaf and pollen DNA from different individual plants as a template.

When taxon-specific sequences revealed polymorphism, degenerate probes were designed. TaqMan probes were dual labeled at the 5′ and 3′ ends with a 6-carboxy-fluorescein group and Black Hole Quencher 1 (Biosearch Technologies Inc, Novato, California), respectively.

Standard and Real-Time PCR Protocols

Standard PCR reactions were performed in a 25-μL final volume, with 50 ng of leaf DNA or 20 ng of pollen DNA, 0.2 μM each dNTP, 0.4 μM each primer, and 1 U of DNA polymerase (HotStartTaq; Qiagen, Hilden, Germany). The amplification conditions were as follows: 15 minutes at 95°C, followed by 35 cycles of 45 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C, and then a final step of 8 minutes at 72°C. Leaf material and pollen collected at 3 and 2 different sites, respectively, were analyzed for each taxon.

Real-time PCR reactions were performed using the LightCycler 480 thermocycler (Roche Diagnostics, Mannheim, Germany) in a 15-μL final volume containing 7.5 μL of LightCycler 480 Probes Master (Roche Diagnostics), 10 ng of leaf DNA or 2 ng of pollen DNA, 0.2 to 0.5 μM each primer, and 0.15 to 0.25 μM specific TaqMan probe. Amplification conditions consisted of 10 minutes at 95°C, 40 cycles of 15 seconds at 90°C, and 1 minute at a specific annealing temperature, then 30 seconds at 40°C (Table 2). The amplification cycle at which sample fluorescence exceeded background, defined as threshold cycle (Ct), was determined using LightCycler 480 software and the fit-point method.[16] Three technical replicates of real-time PCR were performed using pollen and leaf DNA collected at 2 different sites for each taxa. Optimal primer and probe concentrations were established by running a matrix of forward and reverse primers at the same and at unbalanced concentrations. The proper probe concentrations finally ranged from 0.1 to 0.25 μM.

Establishment of Standard Curves and Range of Detection

For the realization of standard curves to be used in the quantification assay, pollen DNA was extracted from approximately 60,000 pollen grains of *Ostrya carpinifolia*, *Betula pendula,* and *Parietaria officinalis.* Standard curves were constructed using a 2-fold serial dilution of taxon-specific pollen DNA in a range from 3,000 to 3 grains, which resembles the number of grains detectable in a typical airborne pollen sample. Three technical replicates were performed for each serial dilution, and each standard curve was repeated at least twice. Standard curves were generated by plotting the logarithmic number of pollen grains against mean Ct values obtained by 3 technical replicates. The limit of quantification (LOQ) was calculated considering the minimum number of pollen grains at which the linearity of the standard curve was maintained.

To check reproducibility, 2 *P officinalis* standard curves were generated using DNA extracted from different pollen samples having the same number of grains. For this purpose, the confidence interval of the slope and the intercepts of the 2 standard curves were calculated and compared. *T* tests and regression analysis were applied to evaluate the comparison between real-time PCR results and microscopic counts. Statistical analysis was performed using R software[17] and Microsoft Excel functions (Microsoft Corp, Redmond, Washington).

RESULTS

DNA Extraction

Good-quality DNA was obtained from leaf tissue of all plant species, and this template was used for the development of a taxon-specific detection assay. The addition of proteinase K and sodium dodecyl sulfate to the cetyl trimethyl ammonium bromide method[13] enabled the isolation of DNA suitable for PCR from free and immobilized pollen. The DNA extraction yield was approximately 30 ng/g of pollen. After 4′,6-diamidino-2-phenylindole treatment, the absence of fluorescent signal on tape pieces confirmed complete DNA recovery from trapped pollen.

Taxon-Specific DNA Sequences

By using the bioinformatics approach, appropriate nuclear gene sequences were identified for *B pendula, Artemisia vulgaris, Olea europaea, Alnus, Fraxinus*, and Cupressaceae; a random amplified polymorphic DNA–derived sequence was selected for *Corylus avellana* (Table 2).

On the other hand, a literature search provided candidate target sequences for Poaceae, *P officinalis,* and *O carpinifolia*. A quite conserved region of the single-copy granule-bound starch synthase gene (*GBSS*) was amplified and sequenced for Poaceae using F-for and K-bac primers based on the study by Mason-Gamer et al.[18] Likewise, the conserved ortholog set marker *At103* was PCR amplified and sequenced using degenerate primers for *P officinalis* and *O carpinifolia* as described by Li et al.[19]

Taxon-specific primer pairs, designed *ex novo* for all identified sequences, generated PCR products that were resequenced and homologous to the reference sequences. Complementary DNA probes were finally designed for *B pendula, A vulgaris, C avellana, P officinalis, O carpinifolia, Fraxinus* species, and *O europaea*. Polymorphisms in the target DNA region were instead observed among plant individuals in the

Table 2. Primer and Probe Sequences and Reaction Conditions Used for Real-Time PCR

Organism	Gene	Primer/ probe	Sequence 5′-3′	Conc, nM	Ta, °C	ID
Betula pendula	*BP8*	Forward	ACGATCGAGTTTTCATCAAACAAA	400	60	Z18891
		Reverse	GACCTTATTGTCTTCACGGTCCTT	400		
		Probe	ATGGAAGAGTTGAAGGTGCGAGGCG	150		
Corylus avellana	*RAPD*	Forward	ATGATTCATTTGGTGAGGAAATGG	400	60	CZ257493
		Reverse	GCATAATCCAAGCCTTTACCCTTTA	400		
		Probe	TTGTGTGCCAAGAAGTTTGCTAAGT	150		
Artemisia vulgaris	Squalene synthase	Forward	GATTGGCACTTTGCATGTCAGTAC	400	60	AF405310
		Reverse	AAAGGCAGTAGAAACATGGTGGAA	400		
		Probe	AATTTTTTGTGTCACCCCATATGAT	150		
Cupressus species	Needly	Forward	GACGATTGGAGACTATGATCTA	500	53	AY988307
		Reverse	ATGCTTCCATTAGGGATTAGC	500		AY988279
		Probe	CTTTCCACAWTGTTCTAAGTAAAATTAATACA	250		
Thuja orientalis	Needly	Forward	GACGATTGGAGACTATGATCTA	400	53	AY988307
		Reverse	ATGCTTCCATTAGGGATTAGC	400		
		Probe	TTTCCACATYGATCTAAATAAAATTASTACAT	200		
Fraxinus species	Phantastica	Forward	TCCCGCCATGGATGAATAAC	400	60	DQ679537
		Reverse	AATCCGGGTTCTGGGTGAAT	400		
		Probe	TAACTCTTTCCCCTTCCGAACCG	200		
Alnus species	*Adh1*	Forward	GCTTTTCTTTTTGGCGTGATG	200	60	AM062702
		Reverse	AAGGCAACGGCAAACATATGT	500		
		Probe	CAGAGAGAASAAGCAGTTTTATGTAT	150		
Olea europaea	Oleosin	Forward	CGATACAGCAGAAAGCACCA	400	53	AY083161
		Reverse	AACACACAGTTCACATACACAA	400		
		Probe	CTTGAAGATGGATGATATAGTACAGA	200		
Poaceae	Waxy	Forward	GCAGGGCTCGAAGCG	400	60	Mason-Gamer et al,[18]
		Reverse	GATCGTGCTCCTBGGCA	400		
		Probe	TTGAACTTSACCACGGCCCTCACC	200		
Parietaria officinalis	*At103*	Forward	TCATCTTCTACGCCACCTCCT	400	64	Li et al,[19]
		Reverse	CTGGCACCAATTCTCGAAGTAC	400		
		Probe	AATCCCGAGTTCCAGTGCTACCCCA	200		
Ostrya carpinifolia	*At103*	Forward	GATTAGATGAAAACAGCCAAGAGAAA	400	60	Li et al,[19]
		Reverse	GGAAAGTAAAAGTGTAACTGGGAATTGA	400		
		Probe	AGCCTAGAAATGAAGTCTAATGATATGAATTG	200		

Abbreviations: Conc, primer and probe concentration; ID, National Center for Biotechnology Information accession number or bibliographic reference; PCR, polymerase chain reaction; Ta, annealing temperature.

groups of *Alnus* species, Cupressaceae, and Poaceae. To meet the required classification level, degenerate probes, which hybridized to DNA pools of different species, were designed in these cases. Owing to single nucleotide polymorphisms found in the target region of the Needly gene (accession No. AY988307 and AY988279) in *Cupressus* species and *Thuja orientalis*, 2 different degenerate probes were designed: 1 for the simultaneous identification of *Cupressus* species and 1 for *T orientalis*. Finally, primers and probes based on the Phantastica gene (accession No. DQ679537), of which several sequences form *Fraxinus excelsior* were publicly available, turned out to amplify *Fraxinus* and *Olea* species. Therefore, the identification assay discriminated at the Oleaceae family level and not at the *Fraxinus* genus level as initially required. Accession numbers (National Center for Biotechnology Information database ID) of target DNA regions, primer pairs, and probe sequences are listed in Table 2.

Real-Time PCR Assay

No differences in primer pair specificity were detected between PCR results of different individuals and between leaf and pollen samples. For *A vulgaris*, *C avellana*, *O europaea*, Oleaceae, and Poaceae, no PCR products were observed when related primers were used with other taxa DNA (Table 3). A weak cross-amplification was instead generated using primers developed for *B pendula*, *P officinalis*, *O carpinifolia*, *Alnus,* and Cupressaceae. However, high specificity was achieved when the same primer sequences were applied in combination with the designed probe in real-time PCR. Taxon-specific PCR amplification occurred with Ct values ranging from 25.73 (*C avellana*) to 35.46 (*Cupressus sempervirens*), with a mean of 30.38 (Table 4).

Standard Curves and Real-Time PCR Assay

Standard curves were realized by analyzing at least 5 serial dilutions of pollen DNA starting from 3,000 grains and

Table 3. Summary of Amplification Results of Conventional PCR

Plant species	Taxa									
	Betula pendula	*Corylus avellana*	*Artemisia vulgaris*	Cupressaceae	*Fraxinus* species	*Olea europaea*	*Parietaria officinalis*	*Ostrya carpinifolia*	Poaceae	*Alnus* species
B pendula	+++									
C avellana		+++					+			+
A vulgaris			+++	+						
Cupressus sempervirens				+++			+	+		
Cupressus arizonica				+++						
Thuja orientalis				+++						+
Fraxinus ornus					+++			+		
Fraxinus excelsior					+++					
O europaea					+++	+++				+
P officinalis							+++			
O carpinifolia								+++		+
Poa annua	+								+++	
Lolium perenne							+		+++	
Anthoxanthum odoratum				+					+++	
Dactylis glomerata									+++	
Phleum pratense	+								+++	
Alnus glutinosa								+		+++
Alnus incana										+++

Abbreviations: PCR, polymerase chain reaction; +++, strong amplification; +, weak amplification.

Table 4. Real-Time PCR Detection of Selected Taxa

Taxon	Cycle threshold, mean (SD)[a]
Betula pendula	28.55 (0.14)
Corylus avellana	25.73 (0.40)
Artemisia vulgaris	33.30 (0.42)
Cupressus sempervirens	35.46 (0.07)
Cupressus arizonica	33.62 (0.29)
Thuja orientalis	34.34 (0.37)
Fraxinus ornus	29.20 (0.21)
Fraxinus excelsior	27.39 (0.43)
Olea europaea	32.42 (0.11)
Olea europaea	29.84 (0.08)[b]
Parietaria officinalis	26.50 (0.22)
Ostrya carpinifolia	26.92 (0.34)
Poa annua	29.51 (0.20)
Lolium perenne	32.76 (0.13)
Anthoxanthum odoratum	32.33 (0.14)
Dactylis glomerata	32.42 (0.2)
Phleum pratense	32.60 (0.06)
Alnus glutinosa	30.35 (0.53)
Alnus incana	28.48 (0.32)

Abbreviation: PCR, polymerase chain reaction.
[a] Cycle threshold of each amplification calculated on 3 technical replicates.
[b] Values for *O europaea* were obtained with family-specific primers and probes.

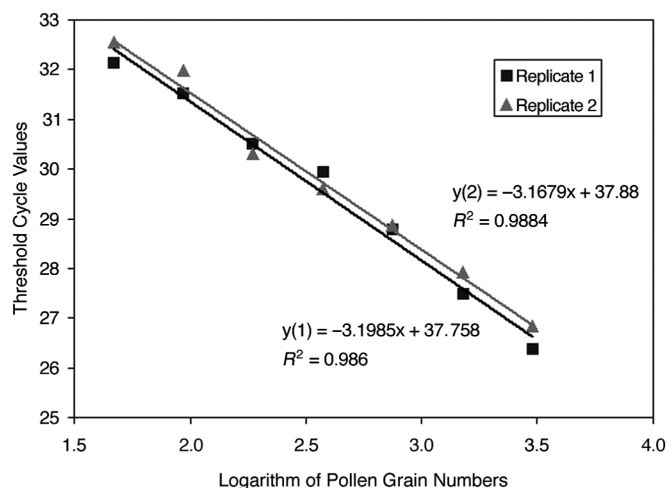

Figure 1. Comparison of standard curves obtained by analyzing 2 *Parietaria officinalis* replicates. Equations and *R* values for the linear regression are shown. Polymerase chain reaction efficiency ($E = 10^{[-1/slope]}$) was 2.1 for both replicates.

achieving LOQ values of 188, 94, and 47 pollen grains for *O carpinifolia*, *B pendula,* and *P officinalis,* respectively. In no cases did the real-time PCR assay reach the LOQ value of 3 pollen grains, probably owing to low DNA amount[20] or low sensitivity of the real-time PCR chemistry.[21]

Standard curves showed a linear regression between input DNA and Ct values in the 2 independent assays, with determination coefficients (R^2) of 0.97 and 0.99 for *B pendula*, 0.99 and 0.99 for *P officinalis,* and 0.95 and 0.96 for *O carpinifolia*. Standard curves realized with DNA pollen samples from different *P officinalis* individuals showed good reproducibility because the 2 linear regression analyses demonstrated well-overlapping 95% confidence intervals for the slope values (replicate 1: −3.56 to −2.65 and replicate 2:

Table 5. Mean Values of Estimated Number of Pollen Grains Analyzed Via Microscopic Counts and Real-Time PCR Quantification

Taxon	No. of replicates	Microscopic counts		Real-time PCR counts		*P* value[a]
		Mean	Standard error	Mean	Standard error	
Parietaria officinalis	5	500	25	389	40.981	.07
	6	1,000	50	911	56.838	
Betula pendula	3	250	7.5	248	42.532	.19
	6	500	15	591	78.319	
	3	1,000	30	1,087	98.376	
Betula pendula mix	3	500	12.5	354	41.94	.45
	6	1,000	25	987	101.96	
Ostrya carpinifolia	2	250	5	250	1.5	.92
	3	500	10	593	65.531	
	7	1,000	20	860	61.329	
	2	1,500	30	1,390	20	
	3	2,000	40	2,127	448.22	
Ostrya carpinifolia mix	3	1,000	25	945	168.33	

Abbreviation: PCR, polymerase chain reaction.
[a] Using the paired *t* test.

−3.75 to −2.91) and highly comparable intercepts (37.88 and 37.76) (Fig 1).

No statistically significant differences were found comparing mean microscope counts with mean estimated real-time PCR pollen grain numbers, analyzing either single taxa or balanced mixes (Table 5). Linear regression applied to mean pollen grain numbers counted using a microscope vs real-time PCR showed an high determination coefficient (R^2 = 0.97), demonstrating good agreement between the estimates provided by the 2 methods (Fig 2). Furthermore, pollen quantification produced comparable values when analyzing mixed- or single-species samples.

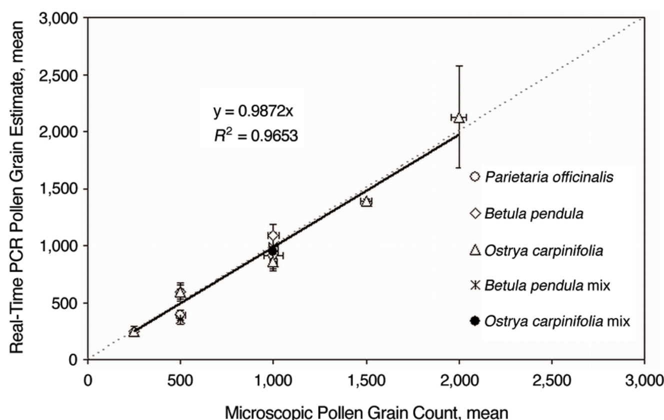

Figure 2. Quantification of pollen grains by real-time polymerase chain reaction (PCR) estimated using taxon-specific standard curves. Pollen grain numbers were estimated from threshold cycle values using the standard curves specific for each taxon, and, subsequently, mean values quantified by real-time PCR analysis were compared with mean microscopic counts. Data were fitted using a linear regression equation (shown in the figure). Error bars represent SE.

DISCUSSION

Pollen recovery is heterogeneous, showing differences according to pollen settling velocity and the distance between sampling sites and the pollen source.[22] The deviation may be even larger in mountainous regions, especially when characterized by an orographic complexity and consequent differences in climatic variables that strongly affect the distribution and development of vegetation. Current allergy warning systems, based on information about airborne pollen content obtained via morphologic microscopy analysis, require qualified operators and result in a time-consuming task. An increase in sampling sites is desirable to improve information regarding allergen exposition, helping individuals with allergy to plan correct management of their disease. Therefore, more efficient approaches to rapid identification of airborne pollen grains have been searched for in other fields. Spectroscopic studies, for example, reported a potential capability of chemical classification through Fourier transform infrared[23] and Raman[24] pollen spectra. Both approaches make an effort in the direction of automating the pollen identification process but show limitations in pollen mixes or pollen quantification analysis.

In the present study, we aimed to develop a real-time PCR method based on TaqMan technology capable of identifying and quantifying a subset of the main allergenic pollen types. Similar approaches have already been applied in many different fields, for example, to the detection and quantification of genetically modified organisms,[25] allergens contained in foods,[26] fungi,[21] nematodes,[27] bacteria,[28] and viruses.[29] A new DNA extraction protocol was successfully developed and applied on a wide spectrum of airborne pollens, even when immobilized on monitoring tape, enabling the use of standard aerobiological samples.

By exploiting DNA sequences available in international databases or sequencing DNA regions *ex novo*, powerful

diagnostic TaqMan assays were developed for each considered taxon. Although plant investigation mainly uses molecular sequences of chloroplast or nuclear ribosomal DNA, in this study, single- or low-copy nuclear genes were preferred because of their higher structure conservation and slower rate of sequence evolution.[30]

The biomolecular method developed in this study allowed successful pollen quantification, statistically comparable with the aerobiological method. The main advantage of the new approach lies in its time requirement: in the time needed for the visual evaluation of weekly samples from a monitoring station by highly specialized personnel, the biomolecular method could manage the analysis of samples from 6 devices.

Results of the analysis of compound samples imply that the quantification assay of a species is not affected by the presence of other DNA templates. Given the high complexity of aerobiological samples, this issue becomes particularly relevant and supports this technique as a promising alternative to the traditional microscopic approach. To our knowledge, this is the first study of the use of real-time PCR for the detection and quantification of pollen grains. Additional efforts are required to lower the minimum number of detectable pollen grains by quantitative PCR, reaching values of the same magnitude order (10^1) as microscope counting.

Future developments could include the use of designed primer pairs and probes in a multiplex reaction to detect and quantify simultaneously different pollen taxa, speeding up the analysis and reducing analytical efforts. In addition, the identified taxon-specific sequences could be a starting point for the application of high-throughput molecular methods, such as hybridization chip or resequencing strategies.

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